

Hanna M. Ollila

# Genetics of Sleep

**Sleep and Comorbidities:  
Connection at the Genetic Level**

RESEARCH



**RESEARCH 96**

Hanna M. Ollila

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## **Sleep and Comorbidities: Connection at the Genetic Level**

### **ACADEMIC DISSERTATION**

To be presented with the permission of the Faculty of Medicine, University of Helsinki, for public examination in Christian Sibelius Auditorium, Psychiatry Center, Välskärinkatu 12, on Friday February 1<sup>st</sup>, 2013 at 12 noon.

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and

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ISBN 978-952-245-812-4 (printed)

ISSN 1798-0054 (printed)

ISBN 978-952-245-813-1(pdf)

ISSN 1798-0062 (pdf)

URN:ISBN:978-952-245-813-1

<http://urn.fi/URN:ISBN:978-952-245-813-1>

Juvenes Print – Tampereen Yliopistopaino Oy  
Tampere, Finland 2013

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*People saying “It cannot be done”  
should not stop those doing it.*



To Otto





## Abstract

Hanna Ollila, Genetics of sleep, Sleep and Comorbidities: Connection at the Genetic Level. National Institute for Health and Welfare. Research 96. 166 pages. Helsinki, Finland 1.2. 2013.

ISBN 978-952-245-812-4 (printed); ISBN 978-952-245-813-1 (pdf)

Sleep is a complex genetic trait with substantial heritability of up to 44%. Sleep problems are increasing in the society and the average sleep duration has been decreasing gradually during the past decades. At the same time the amount of cardiometabolic and psychiatric diseases has steadily increased. The common genetic variants contributing to sleep duration are largely unknown and we still do not know why we sleep. Recent advantages in genetics have provided the scientific community with the tools to elucidate the genes behind complex genetic traits as well. In this thesis, the traditional candidate gene approach as well as genome-wide tools combined with functional analysis were used to study the normal variation in sleep duration (I), consequences of sleep loss (I) and finally the connection of sleep with co-morbid diseases in humans (II-IV).

Genetic variation behind normal sleep duration was studied using genome-wide association (GWAS) (I). The function of the variants was elucidated using RNA expression in population level. In addition, the variants that showed nominally significant association in the follow up sample were studied using RNA expression after experimentally induced sleep restriction (I). The original GWAS did not produce genome-wide significant findings. However, recent studies have shown that part of the association signals reaching only suggestive levels of association may be of biological relevance. We thus followed up the top 32 signals with suggestive evidence for association in a follow-up sample of 6834 individuals. Out of these, three SNPs with point wise  $P < 0.05$  associated with normal sleep duration. The SNPs were located near genes encoding for *Krüppel like factor 6* (*KLF6*), *protein tyrosine phosphatase receptor type U* (*PTPRU*) and between *centidin 1* (*CENTD1*) and *protocadherin 7* (*PCDH7*). *KLF6* variant associated with shorter sleep duration and *KLF6* expression levels associated with shorter sleep duration. Accordingly, experimental sleep restriction increased *KLF6* expression levels. Furthermore, the expression levels of *KLF6* associated with increased slow wave sleep duration. This suggests that the variant in *KLF6* may contribute to normal sleep duration via increased *KLF6* expression and increased sleep intensity (I). However, the findings obtained from the GWAS should be interpreted with caution due to the relatively small number of individuals in the discovery data set and lack of genome-wide significant findings.

The changes in the RNA expression after experimental sleep restriction revealed cellular activation of immune reaction and down regulation of cholesterol and lipid metabolism (I and unpublished). Sleep restriction may convey its proatherogenic

effects via low grade inflammation and imbalance of metabolism, which previously have been related to cardiometabolic diseases. These changes may help to cope with short term sleep debt but are likely to induce pathological changes in the long term.

In order to study the genetic connection of sleep duration with somatic diseases, the previously identified genome-wide significant variants associating with blood lipid levels were selected for association analysis with sleep duration. Two variants near *Tribbles1* (*TRIB1*) associated with sleep duration independently from blood lipid levels (II). In addition, *TRIB1* RNA expression levels were increased after experimental sleep restriction (II). The findings suggest that there are common genetic variants, such as those in *TRIB1* that may regulate both sleep duration and blood lipid levels. The observations of shared genetic regulation both in RNA expression and in genetic level may partially explain why sleep duration is related to cardiovascular diseases.

Finally, we evaluated the connection between psychiatric diseases and sleep. A common challenge when studying psychiatric phenotypes is the broad spectrum of disorders. In addition to the psychiatric diagnosis we studied endophenotypes such as insomnia, diurnal preference, seasonality and performance in cognitive tests and classified the study subjects in order to get a phenotypically, and also potentially a genetically more homogenous study population. One of the key molecules in homeostatic sleep regulation is adenosine. Its levels have recently also been related with depression. We found adenosine-related genetic polymorphisms that associated with depression (III). The findings suggest that the connection of sleep with psychiatric diseases may be partially explained by common genetic factors that mediate both sleep and psychiatric diseases. This hypothesis was further tested with patients suffering from bipolar disorder (BD). In BD, circadian stress such as jet lag or shift work can induce manic episodes. We thus tested if the same genetic variants associate with BD and with circadian and seasonal phenotypes as well as with cognitive task performance. We found variants in *Cadherin 7* (*CDH7*) to associate with both sleep phenotypes and with BD. Interestingly, the variants predisposing to BD associated with better performance in visual processing, suggesting an evolutionary advantage for having the risk allele for BD. Together the findings suggest a shared genetic background for regulation of sleep with somatic and psychiatric disorders. It is important to identify the genetic factors contributing to sleep, together with somatic and psychiatric diseases. The knowledge of biological functions creates a strong basis for developing efficient treatments for sleep and psychiatric disorders.

**Keywords:** Sleep, polymorphism, RNA expression, cardiovascular disease, mood disorders

## Tiivistelmä

Hanna Ollila, Unen yhteys mielialan ja aineenvaihdunnan häiriöihin sekä taustalla vaikuttavat geneettiset tekijät. Terveyden ja hyvinvoinnin laitos. Tutkimus 96. 166 sivua. Helsinki, Finland 1.2.2013.

ISBN 978-952-245-812-4 (painettu); ISBN 978-952-245-813-1 (pdf)

Uni on monitekijäinen geneettinen ominaisuus, jolla on merkittävä perinnöllinen osuus, 44%. Viime aikoina uniongelmat ovat yleistyneet yhteiskunnassa ja keskimääräinen unen pituus on vähentynyt. Samaan aikaan Suomessakin yleiset sydän- ja verisuonitaudit, tyypin 2 diabetes sekä mielialahäiriöt ovat lisääntyneet. Yleiset perinnölliset tekijät sekä yksittäiset geenit, jotka vaikuttavat uneen, ovat suurelta osin vielä tuntemattomia. Viimeisen kymmenen vuoden aikana kehitetyt uudet geneettiset tutkimusmenetelmät ovat kuitenkin luoneet työkaluja, joilla myös monitekijäisten ominaisuuksien tutkiminen on mahdollista.

Tässä väitöskirjatyössä tutkittiin normaaliin unen pituuteen vaikuttavia yleisiä perinnöllisiä tekijöitä suomalaisessa väestössä käyttämällä koko perimän kattavia yhden nukleotidin geenimerkkejä (I). Löydökset varmennettiin toistoaineistossa ja niiden toimintaa tutkittiin kokeellisissa malleissa populaatiotasolla sekä vapaaehtoisilla laboratorio-olosuhteissa (I). Lyhyen unen terveysvaikutuksia tutkittiin solutasolta lähtien käyttäen koko genomin RNA-ilmennyskirjastoa kokeellisessa ja kontrolloidussa laboratorioympäristössä. Havaittujen yksittäisten geenien merkitys väestötasolla mitattiin RNA-ilmentymisellä, jotka yhdistettiin mittauksiin unen pituudesta (I ja julkaisemattomat havainnot). Lopulta tutkimme unen laadun sekä vuorokausirytmien yhteyttä somaattisiin sairauksiin sekä mielialahäiriöihin perinnöllisellä tasolla: liittyvätkö mielialahäiriöissä ja somaattisissa sairauksissa toimivat geenit geneettisellä tasolla unen pituuteen tai laatuun.

Havaitsimme, että *KLF6*, *PTPRU* sekä *CENTD1-PCDH7* geenien läheisyydessä olevat geenimuodot liittyvät normaaliin unen pituuteen. Toiminnallinen analyysi paljasti lisäksi, että *KLF6*-geenimuoto liittyy myös *KLF6*:n ilmentymiseen ja lyhytunisilla oli korkeampi *KLF6* ilmentyminen populaatiotasolla. Kokeellisessa univajeessa löytö toistui ja univaje lisäsi *KLF6*:n ilmentymistä ja lisäksi liittyi suurempaan hidasaaltouden määrään. Tuloksemme viittaavat, että *KLF6*-geenimuoto vaikuttaa unen pituuteen *KLF6*:n ilmentymisen sekä hidasaaltouden kautta (I).

RNA-ilmentymistyössä havaitsimme solutasolla puolustusreaktioiden käynnistymisen univajeen seurauksena sekä aineenvaihduntatasapainon muuttumisen matalammaksi (I ja julkaisemattomat havainnot). Pitkittynyt matalan tason puolustusvaste on tunnettu sydän- ja verisuonitautien riskitekijä. Tuloksemme osoittavat, että univaje saattaa altistaa sydän- ja verisuonitauksille immuunipuolustuksen käynnistymisellä sekä aiheuttamalla aineenvaihdunnan häiriöitä.

Lisäksi etsimme geenimuotoja, jotka vaikuttavat sekä normaaliin uneen että altistavat somaattisille sairauksille. Havaitsimme, että kaksi *TRIB1*:n lähellä olevaa

geenimerkkiä liittyvät veren rasva-aineenvaihdunnan lisäksi normaaliin unen säätelyyn (II). Kokeellinen univaje lisäsi myös geenin luentaa (II).

Lopuksi etsimme geenimuotoja, jotka liittyvät mielialahäiriöihin. Mielialahäiriöissä potilaiden ilmiäisy on usein laaja. Jotta saisimme ilmiäisun selkeämmäksi, tutkimme myös tautiin liittyviä ilmiäisiä, kuten unettomuutta, väsymystä, vuorokausirytmiä, kognitiivisia testejä sekä vuodenaikaisrytmiä. Löysimme masennukseen liittyviä geenimuotoja perinteisistä uneen liittyvistä adenosiniaineenvaihduntaa säätelevistä geeneistä (III). Lisäksi löysimme geenejä kaksisuuntaisessa mielialahäiriössä, jotka liittyvät kaksisuuntaisen mielialahäiriön lisäksi vuorokausi- ja vuosirytmin joustavuuteen sekä kognitiivisiin testeihin (IV). Havaitimme, että samat geenimuodot yllättäen paransivat suoritusta kognitiivisissa testeissä, vaikka ne liittyivät kaksisuuntaiseen mielialahäiriöön. Tuloksista voidaan päätellä, että taudille altistavat variantit voivat olla edullisia jollekin toiselle ominaisuudelle. Löydökset myös selittävät, miksi ne ovat säilyneet valinnasta huolimatta. Näistä tuloksista voimme päätellä, että uni ja mieliala sekä aineenvaihdunta ovat kytkeytyneet jo perinnöllisellä, yksittäisten geenimuotojen tasolla toisiinsa.

Unen kanssa yhteisvaikuttavien geenien toiminnan tunteminen on tärkeää sairauksien synnyn ymmärtämisen kannalta. Biologisten reittien kartoittaminen luo myös vahvan pohjan uusien lääkemolekyylien kehittämiseksi.

Avainsanat: uni, mielialahäiriöt, sydän- ja verisuonitaudit, genetiikka, SNP

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## Abbreviations

ARAS	Ascending reticular arousal system
ATP	Adenosine-5'-triphosphate
bp	Base pair
bHLH	Basic helix-loop-helix
BD	Bipolar disorder
BP	Blood pressure
BMI	Body mass index
cDNA	Complementary DNA
CDH7	Cadherin 7
CENTD1	Centidin 1
CHD	Coronary heart disease
CIDI	Composite International Diagnostic Interview
CNV	Copy number variation
CRP	C-reactive protein
dbSNP	The Single Nucleotide Polymorphism database
DILGOM	Dietary Lifestyle and Genetic determinants of Obesity and Metabolic Syndrome
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual
DZ	Dizygotic
EEG	Electroencephalography
EMG	Electromyography
ENCODE	The Encyclopedia of DNA Elements
EOG	Electro-oculography
eQTL	Expression quantitative trait locus
GABA	Gamma-aminobutyric acid
GH	Growth hormone
GWA study	Genome-wide association study
HDL-C	High-density lipoprotein cholesterol
HGP	Human Genome Project
HLA	Human leukocyte antigen
HPA-axis	Hypothalamic-pituitary-adrenal axis
HWE	Hardy-Weinberg equilibrium
IBD	Identical by descent
IBS	Identical by state
IL	Interleukin



kb	Kilobase
KLF6	Krüppel like factor 6
LD	Linkage disequilibrium
LDL-C	Low-density lipoprotein cholesterol
MAF	Minor allele frequency
MCH	Melanin concentrating hormone
mRNA	Messenger RNA
MZ	Monozygotic
NREM	Non-REM
PCR	Polymerase chain reaction
PCDH7	Protocadherin 7
PSG	Polysomnography
PTPRU	Protein tyrosine phosphatase type U
QC	Quality control
REM	Rapid eye movement
RLS	Restless legs syndrome
RNA	Ribonucleic acid
SCN	Suprachiasmatic nucleus
SE	Standard error
SAD	Seasonal affective disorder
SNP	Single nucleotide polymorphism
SWA	Slow wave activity
SWS	Slow wave sleep
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triacylglycerols
TRIB1	Tribbles 1
tRNA	Transfer RNA
VLPO	Ventrolateral preoptic nucleus
VNTR	Variable nucleotide tandem repeats
YF	Young Finns

## List of Original Papers

- I     **Ollila HM**, Kettunen J, Pietiläinen O, Aho V, Silander K, Perola M, Partonen T, Kaprio J, Salomaa V, Raitakari O, Lehtimäki T, Sallinen M, Härmä M, Porkka-Heiskanen T and Paunio T 2012. Genomics of sleep length – A genome-wide association study of sleep duration. Submitted manuscript
  
- II    **Ollila HM**, Utge S, Kronholm E, Aho V, Van Leeuwen W, Silander K, Partonen T, Perola M, Kaprio J, Salomaa V, Sallinen M, Härmä M, Porkka-Heiskanen T and Paunio T 2012. TRIB1 constitutes a molecular link between regulation of sleep and lipid metabolism in humans. Translational Psychiatry(2012) 2, e97
  
- III   **Gass N, Ollila HM**, Utge S, Partonen T, Kronholm E, Pirkola S, Suhonen J, Silander K, Porkka-Heiskanen T, Paunio T. Contribution of adenosine related genes to the risk of depression with disturbed sleep. J Affect Disord. 2010 Oct;126(1-2):134-9.
  
- IV   **Soronen P, Ollila HM**, Antila M, Silander K, Palo OM, Kieseppä T, Lönnqvist J, Peltonen L, Tuulio-Henriksson A, Partonen T, Paunio T. Replication of GWAS of BD: Association of SNPs near CDH7 with BD and visual processing. Mol Psychiatry. 2010 Jan;15(1):4-6.

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# 1 Introduction

Sleep is common to all animals. It has inspired both artists and scientists for centuries due to its mystical and enigmatic nature, and still despite considerable efforts of the scientific community, it is unknown why we sleep. Insufficient sleep has a large impact on public health and economy. Insufficient sleep or poor sleep quality are both risk factors and co-morbidities for psychiatric and cardiometabolic diseases such as type 2 diabetes mellitus (T2DM), cardiovascular disease, coronary heart disease and obesity (Cappuccio et al., 2010a, Cappuccio et al., 2010b). It has been estimated that poor sleep accounts for \$1967 expenses in productivity per employee every year in USA alone (Rosekind et al., 2010). During the past three decades the mean sleep duration has decreased on estimate more than thirty minutes (Tune, 1968, Kronholm et al., 2008). At the same time, the prevalence of cardiometabolic diseases has steadily increased. Twin studies (Paunio et al., 2009, Watson et al., 2010), genome-wide RNA expression studies (Mackiewicz et al., 2007) and genome-wide association studies (GWA study) (Ingelsson et al., 2010) have all pointed toward a genetic connection between sleep, metabolism and psychiatric diseases.

During the past decade, the understanding of the human genome has evolved from solving the sequence of only a few individuals during the early days of the human genome project (Lander et al., 2001, Venter et al., 2001), into large scale genome-wide gene polymorphism mapping and involving over a hundred thousand study subjects or sequencing studies aiming at several thousand individuals (The 1000 Genomes Project Consortium, 2010). The development of technology, statistical methods and above all understanding of the genomic landscape, have yielded tools that were not available before to study complex genetic traits, such as sleep. In several, especially cardiometabolic traits, this progress has resulted in an increasing amount of disease loci and given important new information on human physiology (Ingelsson et al., 2010, Teslovich et al.). The heritability of sleep duration is also substantial, 44% (Partinen et al., 1983). This is probably due to the strong heritability of electroencephalography (EEG) traits, which is over 96% (De Gennaro et al., 2008).

Ten years ago, only a few genes and genetic variants for complex genetic traits such as sleep were known. In terms of sleep research, the genetic studies are needed to solve the molecular mechanisms in sleep regulation and the connection between sleep and comorbid diseases. In effect, studies on sleep duration, chronotype, sleep quality and EEG traits have now entered the phase of large-scale genetic studies based on GWA studies or genome-wide RNA expression studies. This thesis aims to use the traditional candidate gene approach as well as genome-wide tools combined with functional analysis to study the normal variation in sleep duration,

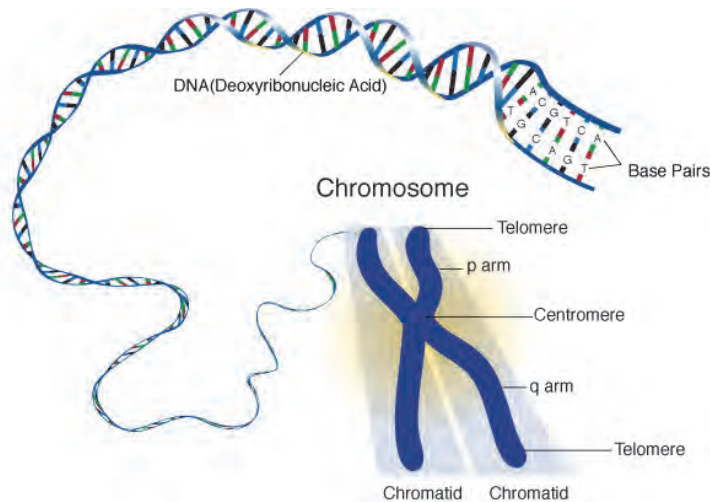
consequences of sleep loss and, finally, the connection between sleep and comorbid diseases in humans on a genetic level.

## 2 Review of the Literature

### 2.1 Genetic Architecture of Complex Traits

#### 2.1.1 DNA

The human genome consists of roughly 3.2 billion base pairs (bp) of deoxyribonucleic acid (DNA) and it carries most of the information needed for inheritance. DNA is stored packed around histone molecules that support the structure. It is divided into chromosomes that are located in the nucleus of every cell. The structure of DNA is presented in Figure 1. DNA has two basic elements: a sugar phosphate backbone and a nucleobase which is attached to the sugar. The order of the bases converts the information in the form of genes. There are four common bases in DNA that form the DNA sequence: adenine (A), guanine (G), cytosine (C) and thymine (T). Most cells have DNA to store the information but RNA viruses rely on RNA for information storage as well as circulating red blood cells that only have RNA transcripts and protein products needed for cell function. Altogether, 22 autosomes and 2 sex chromosomes (X and Y) exist in humans and the genome is diploid in nature containing two copies of each chromosome, except for germ line cells that are haploid.



**Figure 1.** DNA is packed in chromosomes (<http://www.genome.com>). DNA consists of a double helix made of deoxyribonucleic acid. The variable base region consists of adenine (A), thymine (T), cytosine (C) and guanine (G). A forms a base pair with T and C forms a pair with G. DNA is bent into a double helix. Histone molecules stabilize the structure and the DNA is packed in chromosomes. Chromosomes are located in the nucleus of the cell.

All individuals are 99.9% identical based on their DNA sequence and thus only 0.1% of the DNA sequence is responsible for genetic differences (Przeworski et al., 2000, Reich et al., 2002). Most of the genetic variation in the DNA that affects the phenotype has been thought to be located in genes. The estimated number of genes in human the genome has come down from 100 000 to 20 000 (Carninci and Hayashizaki, 2007, Lander et al., 2001, Venter et al., 2001). Today it is thought that it is both the complex interplay between genes and networks formed by the genes rather than only the amount of specific gene products or individual genetic variants that affect the studied traits in humans (Chalancon et al., 2012). It was also recently discovered that most of the individual variants can have a functional role either in regulating chromatin structure, RNA transcription, histone modification or transcription factor binding, discussed below (Bernstein et al., 2012).

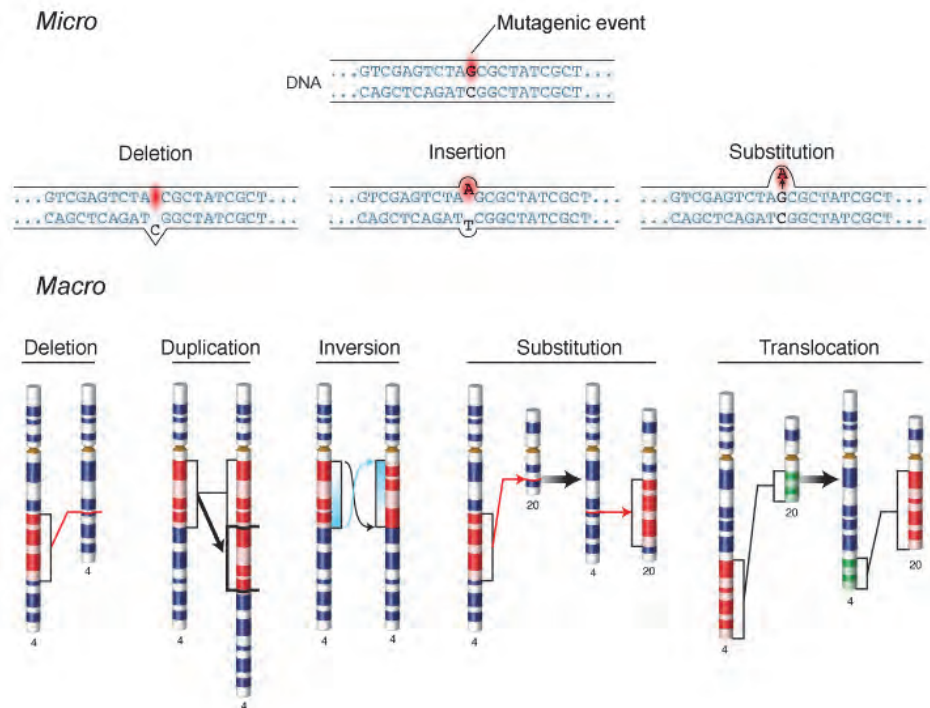
#### *2.1.1.1 Variation in DNA*

The 0.1% variation in the sequence can occur in several forms between individuals (Figure 2). Single nucleotide polymorphisms (SNPs) are one base pair differences between individuals in a given chromosomal location (locus). SNPs are usually bi-allelic, meaning that two of the four different nucleotides can occur in a bi-allelic locus. A certain combination of SNP-alleles in one region forms a haplotype and variation can occur also at the haplotype level. Individuals with different haplotypes have a different set of SNP-alleles forming the haplotype (HapMapProject, 2005).

The genomic DNA also contains repeat sequences of short nucleotide sequences that vary in length between individuals. These variable nucleotide tandem repeats (VNTR) are used for example in genetic fingerprinting and forensics, and can be further divided into several classes. The most common classes are microsatellites and minisatellites. Microsatellites contain short tandem repeats and short sequence repeats, whereas minisatellites have more than ten base pairs.

Nucleotide sequence differences of more than 1kb at a given locus are called copy number variations (CNV) (Fredman et al., 2004, Iafrate et al., 2004, Sebat et al., 2004). In addition, there are five major forms of variation that can be used to categorize either SNPs or CNVs: 1) deletion, where the individual is missing a part of the genome 2) duplication, where there is an increased amount of copies of a given sequence 3) substitutions and in case of only CNVs 4) inversion, where the sequence of DNA is inverted and 5) translocation, where the DNA fragment is located or copied into a different region (Figure 2). Large CNVs are commonly found especially in cancers. Genome-wide studies have found these variants also contribute to other common complex diseases, especially in the field of psychiatric genetics (Kirov et al., 2012, Stefansson et al., 2008).

New genetic variants are constantly introduced to the human genome on average at the rate of  $10^{-8}$  base pairs per generation (The 1000 Genomes Project Consortium, 2010) and each individual carries approximately 250 to 300 loss-of-function variants (The 1000 Genomes Project Consortium, 2010). The genetic variations and new random mutations occurring even today have enabled the survival of the human race in evolution.



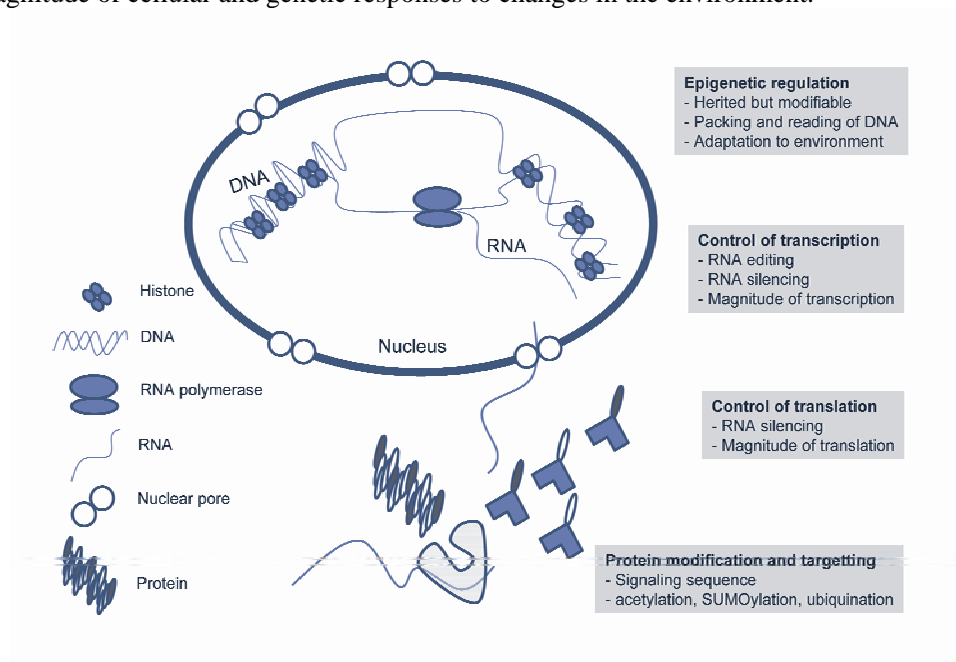
**Figure 2.** Variation in DNA (<http://www.genome.com>). Variation in DNA can occur in small scale (Micro) where individual bases are changed into other bases or in large chromosomal scale (Macro) where several nucleotides or even chromosomal regions are mutated or differ between individuals.

### 2.1.2 RNA Expression and Gene Networks – From Gene to Function

All proteins in an organism are produced based on a genetic code. The coding sequence of DNA is first *transcribed* into an intermediate product, messenger RNA (mRNA) that is then used as a template for transfer RNA (tRNA), which adds a correct amino acid in the growing protein polypeptide chain thus *translating* the information into a protein (Figure 3). Proteins are then further modified with



posttranslational modifications and signal sequences that mark the destination for the proteins. DNA is similar in almost all cells in the body, except for white blood cells, where the DNA sequence in the regions encoding pathogen recognizing proteins is actively modified by adding or deleting nucleotides in order to create larger variability of receptors that can detect pathogens. Another exception is germline, which is haploid. The divergence in cellular phenotypes is mediated mainly through changes in the amount of enzymatically functional RNAs and proteins. Similarly, individual SNPs and differences in the genetic code mediate their effect on the phenotype largely due to differential gene expression and thus different amounts of produced proteins (Nica et al., 2010, Nicolae et al., 2010). The normal variation only rarely produces dysfunctional proteins but affects the magnitude of cellular and genetic responses to changes in the environment.



**Figure 3. Regulation from DNA to protein level (© Hanna Ollila).** The DNA in the nucleus of a cell is first transcribed into mRNA, which is transported to the cytoplasm. The mRNA molecule is then used as a template for tRNA that adds new amino acids into the growing amino acid chain. Once the amino acid chain has been completed the new protein is synthesized. All these events are regulated and can have an effect on the phenotype.

There are a number of elements in the DNA sequence that affect the magnitude and precision of the transcription. Promoter sequences, located typically upstream of the gene, contain binding sites for transcription factors and RNA polymerase that mediate the transcription of DNA to RNA. Transcription factors can modulate the

magnitude of expression either by enhancing the binding and transcription of RNA polymerase or repressing the transcription and binding of the polymerase. Several transcription factors have been identified for sleep regulation. These include the CLOCK and BMAL transcription factors that dimerize into molecular complex. This binds a basic helix-loop-helix (bHLH) transcription factor binding site on the promoter of their target genes and thereby helps to activate their transcription (Lee et al., 2001).

In addition, the modifications of a DNA molecule can affect the magnitude of RNA expression. These covalent modifications are made onto the histone molecules supporting the structure or into the DNA itself and are called epigenetic modifications. The most studied forms of epigenetics are methylation and acetylation. The modifications in DNA are called epigenetic modifications since they do not affect the coding nucleotide sequence but the activity of the genes. Epigenetic modifications can also be inherited from one cell generation to the next (Henikoff and Shilatifard, 2011, Law and Jacobsen, 2010). Similarly, the density of DNA packing and the epigenetic modifications are related to the tissue specific expression of genes and may explain part of the difference of gene expression levels in the central nervous system and in the peripheral tissues. Epigenetic modifications are a way to react to the environment and change the activity of genes by making structural changes in the DNA without affecting the coding sequence (Kota and Feil, 2010, Law and Jacobsen, 2010, Okano et al., 1999, Reik, 2007). In the field of psychiatric genetics for example, it has been shown that working in a stressful environment is related to lower methylation of serotonin transporter *SLC6A4* and thus potentially higher expression levels (Alasaari J, 2012). Interestingly, a familial form of narcolepsy has been found to be caused by a mutation in gene encoding for DNA methylation transcription factor 1 (DNMT1). Such a mutation is likely to have a wide-range effect on overall methylation at the genome level (Devlin et al., 2010, Winkelmann et al., 2012).

#### 2.1.2.1 *Transcriptional Networks*

The individual genes in an organism only rarely carry out their function alone but are linked together into pathways and networks where several genes have a joint effect on a biological function.

Genes can be clustered in pathways based on their function, like metabolic pathways, signal transduction pathways or regulatory pathways. Joint efforts from Gene Ontology (Ashburner et al., 2000) and KEGG Consortiums (Kanehisa et al., 2004) have clustered genes into the pathways based on their biological functions. Similarly, SNPs can be clustered into pathways based on the annotations that define either the closest gene or target gene of whose expression is affected by a SNP. An example of pathways relevant for human diseases is the signal transduction pathway NF- $\kappa$ B signalling cascade that has been shown to be important in autoimmune diseases, especially in Crohn's disease. In this disease, aberrant integration of the

pro-inflammatory and anti-inflammatory cytokine pathways is partially responsible for disease progression (Abbott et al., 2004).

Most genes or their protein products are integrated in the cells. Integrative networks can include both inhibitory and activating parts so that the sum of the network, such as the overall changes in the expression levels, is more precisely connected to the biological phenomena than the action of the individual proteins or pathways alone. Cells are fine-tuned to receive information from environmental challenges and responses by taking into account the availability of cellular resources. A single gene can be part of several networks. This makes it possible to integrate information in the network and create interactions between the pathways. Another benefit from networks is that they affect the biological responses by combining responses from two or more pathways (Ashburner et al., 2000).

### 2.1.3 The Human Genome

The beginning of the 21<sup>st</sup> century has been a success story for human genetic research. The sequence of the human genome was first elucidated by the Human Genome Project (HGP). The aim of the project was to characterize the DNA sequence of the human genome. The original genome was based on the sequence of four individuals, two males and two females, and was published by the HGP and by Celera, a commercial company, in 2001 (Lander et al., 2001, Venter et al., 2001).

The HGP was completed in 2003, as well as the characterization and the publication of the single nucleotide polymorphisms and their allele frequencies in the dbSNP database, making it possible for independent researchers to access and use the genome data (International Human Genome Sequencing Consortium, 2004, Sachidanandam et al., 2001, Sherry et al., 2001).

The next findings came in the form of haplotypes. It was shown that there is a high linkage disequilibrium (LD) in the human genome i.e. markers in the vicinity of each other are more often inherited together than what would be expected by chance (Pritchard and Przeworski, 2001). A few years later, the international HapMap project published the first haplotype map of the human genome (The International HapMap Consortium, 2005). The goal of the HapMap project was to determine the common patterns of DNA sequence variation in humans (The International HapMap Consortium, 2003). Altogether 3.1 million SNPs and their allele frequencies were characterized (Frazer et al., 2007, The International HapMap Consortium, 2005). The HapMap study sample comprised of individuals from four different populations and the main findings from this project were that the genome is organized into recombination hotspots that create LD blocks. Moreover, the haplotypes were found to be shared across different populations (The International HapMap Consortium, 2005, Jakobsson et al., 2008). Together these findings enabled a precise design of genotyping that is also utilized by the GWAS platforms today. This means that variation in a given region can be studied by genotyping only the

tagging SNPs (tagSNP). These tagSNPs and their haplotypes will provide information of all the common variation in the region even though the measured tagSNPs are only rarely the causative variants that associate with the trait (The International HapMap Consortium, 2005).

A deeper coverage based on the sequencing of the human genome was provided in 2010 by the 1000 Genomes Project (1000 Genomes, <http://www.1000genomes.org/>) (The 1000 Genomes Project Consortium, 2010). The original goal of this project was to characterize genetic variation that is present in more than 1% of the population. The 1000 Genomes project was based on low coverage sequencing of the study participants from five different populations from West Africa, Europe, East Asia, South Asia and the Americas. The study expanded the understanding of the sequence variation of SNPs and CNVs in the human genome in large scale and also studied the rare variation in the genome at individual and population levels from five different populations. Altogether 15 million SNPs were discovered, of which, two thirds were already known by dbSNP. In addition, 1 million short CNVs and 20,000 structural variations were discovered. The genetic variation was lowest near the transcription start site of the genes suggesting, that selection reduces variation at these sites. This finding supported the earlier hypothesis that genes are most vulnerable for large changes in the DNA sequence since they encode the functional proteins (The 1000 Genomes Project Consortium, 2010).

As the early breakthroughs in human genetics by HGP found, rather surprisingly, that most of the genome is non-coding DNA and that exons comprise only 1% of the genome (Venter et al., 2001), the rest of the genome was called simply “junk DNA”. For some time junk DNA was considered to have no or just minor biological relevance. However, the ENCODE Project (Encyclopedia of DNA Elements) aimed at characterizing functional elements in the DNA sequence. In September 2012 a number of roles with biological significance were found for the junk DNA by the ENCODE Project. The new studies found biological functions in regulation chromatin structure, RNA transcription, histone modification, or transcription factor binding for more than 80% of the genome (Bernstein et al., 2012). This also shed light on association studies as many of the SNPs that are known to affect biological traits are also located outside the protein coding sequence of DNA. Similarly the number of protein coding genes was estimated at 23,000–40,000 based on the original papers and has now come down closer to 20,000, suggesting that there is much more than just individual genes to look for in the genome (Carninci and Hayashizaki, 2007, Lander et al., 2001, Venter et al., 2001).

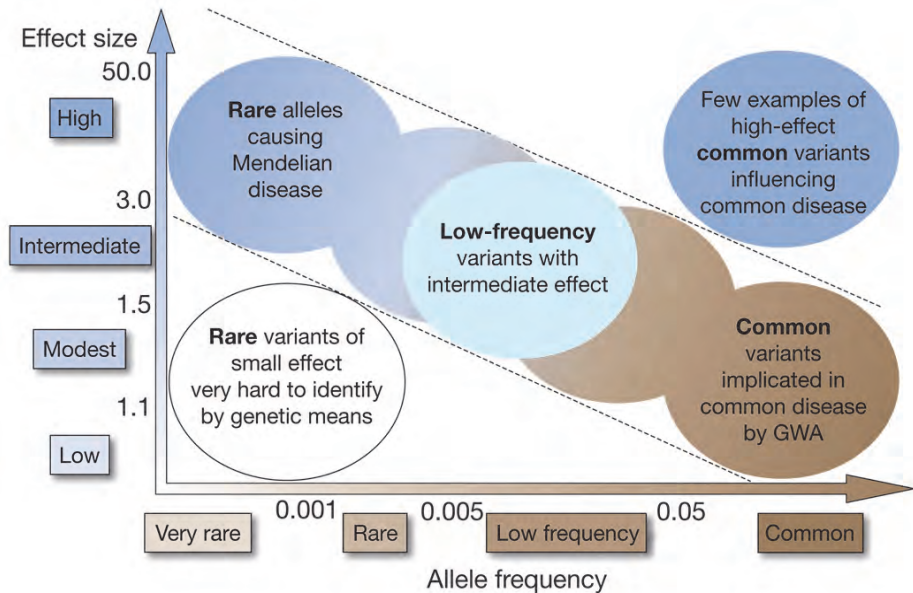
Genetic variation can take place both between populations and within a population. Studies in population genetics have evidenced a larger within population variation than variation between populations. Even though most of the variation is neutral, some parts of the genome are under strong selection (Voight et al., 2006).

There are also a large amount of SNPs that show different allele frequencies in different populations or are population specific. It may be that those SNPs that differ between populations, could imply local adaptation (The 1000 Genomes Project Consortium, 2010).

In the Finnish population, which is relatively isolated from the rest of the European populations, the amount of genetic variants is smaller and there is stronger LD between markers i.e. they are more likely to be inherited together. Nonetheless, there is quite large variation between different geographical regions within Finland (Jakkula et al. 2008).

#### **2.1.4 Complex Genetic Traits**

In polygenic traits many variants are thought to contribute to phenotypic variation. In these traits, a common marker, common disease view prevails. It is thought that a number of relatively common variants of over 1–5% frequency SNPs with low effect size contribute to the genetic component in common diseases. These variants would have such a low individual effect size that they would have remained in the population despite selection (Figure 4). Even though these variants have relatively small effect themselves, they present a significant effect when studying these variants in the scale of the population. This is due to the fact that many individuals carry the low effect risk variants that predispose to a disease or affect a trait (Risch and Merikangas, 1996). In contrast, disease markers with high effect on the trait would be under negative selection and thus become rarer (Lander, 1996, Chakravarti, 1999).



**Figure 4. Common disease, common variant.** In the common disease common variant hypothesis it is thought that the markers present in over 5% of the population account for phenotypic variation. These markers have low effect size ( $OR < 2.0$ ). In the rare variant hypothesis it is thought that rare variants present in less than 0.5% of the population with higher effect size contribute to the phenotypic variation ( $OR > 2$ ). In addition, variants with 0.5%–5% in the population can have intermediate effect on the phenotypic variation. Reprinted with permission. Manolio et al. *Nature* 2009.

The rare variant hypothesis states that a large amount of rare population specific genetic variants with large effect size account for a larger part of genetic variation within a trait, and while the common variants only account for a minority of the variation (Gibson et al., 2012). Both common and rare variants are now thought to contribute to the variation in complex traits. In addition, variants present in 0.5%–5% of the population can have intermediate effect on phenotypic variation.

### 2.1.5 Methods for Studying the Genetic Component of a Disease

Population genetic studies aim at finding the genetic background of common traits and diseases at the level of population. The first step in studying genetic traits is to show using family, twin or adoption studies that the trait has a genetic component that it is inherited. It should be noted that heritability estimates do not tell the number of genes or variants that associate with the trait but how much of the variation is explained by genetic factors. The second step of analysis is to elucidate which genetic markers associate with the trait.

### 2.1.5.1 *Twin Studies*

Twin studies can dissect the variation caused by genetic factors compared to environmental factors, since monozygotic twins (MZ) share nearly 100% the same DNA, while dizygotic twins (DZ) as well as siblings, share 50%. The environment of twins is also more similar than that of normal siblings since they develop at the same time in the uterus. As genes often interact with the environment, adoption studies are also valuable for geneticists and can distinguish the effect of postnatal environmental factors more precisely. In classical twin studies the covariance for a trait in DZ twins is compared to the covariance in MZ twins. Genes can have an effect on a studied trait together with the environment. Such interactions can be studied using gene environment interaction models. In classical twin studies it is estimated that these interactions are absent.

### 2.1.5.2 *Family Studies and Linkage Studies*

Family studies can be used for estimating the model of inheritance: for example whether a disease is inherited as a dominant or recessive trait. As most of the common complex traits are a sum of several genetic factors, they are polygenic. The individual genetic variants have their own model of inheritance. For monogenic traits, only one gene is necessary but also sufficient to cause a disease. However, even for monogenic traits there is variability in the phenotype. The proportion of affected individuals compared to all individuals carrying a disease variant is called penetrance. For variants with high penetrance and one contributing genetic variant, it is usually relatively feasible to define the model of heritability in contrast to complex polygenic diseases.

Families can be studied using linkage analysis, where the segregation of a marker is estimated together with the disease. However, linkage analysis requires the individual variants to have relatively large effect size (Risch and Merikangas, 1996). Normally, association analysis is used to detect low effect variants in unrelated individuals, but it can also be applied to study relatives.

In addition to the diseases themselves, heritable traits named endophenotypes can be used in the search for the genetic background of a psychiatric trait, such as cognitive performance in bipolar disorder or schizophrenia (Clark et al., 2005, Lenox et al., 2002). These typically quantitative traits can also be measured in unaffected family members. They are “measurable components unseen by the unaided eye along the pathway between disease and distal genotype” (Gottesman and Gould, 2003). Endophenotypes are inherited together with disease phenotypes and can be regarded as latent liability to the disease (Lenzenweger, 1999). Endophenotypes can be used to study the unaffected relatives that may carry some of the same predisposing variants common to the disease and the endophenotype. Including unaffected individuals with endophenotypes increases the sample size and thus power. It is thought that disease variants for some psychiatric traits remain in

the normal population due to the beneficial effects and that only accumulation of many variants will predispose to these complex diseases.

#### *2.1.5.3 Association*

Behind association studies there is a hypothesis that the studied marker is in high LD with the causative variant. Association analysis is used in case-control studies and cross-sectional population studies. It tests whether a variant has a different frequency in cases compared to controls. In case of a quantitative trait, it is estimated if the variant is becoming more frequent toward either end of the scale.

Logistic regression models are used for dichotomous traits while linear regression models are mostly used for quantitative traits. For practical reasons the model of inheritance in genome-wide and candidate gene studies for a single risk variant are usually estimated to be additive with no genetic interactions. Where cross-sectional studies can detect associations and risks between a marker and a trait, prospective studies can find causal relationships between them. Additional study settings include experimental settings performed in controlled environments or requiring an intervention. Experimental studies are abundantly used in sleep research where for some of the traits, like polysomnography, overnight recordings are needed.

#### *2.1.5.4 Genome-wide Association*

Association analysis in a genome-wide scale was made possible by HGP. In addition, the annotation of SNPs and their LD structure by HapMap and dbSNP projects made it feasible to design SNP panels that covered most of the variation in the genome. The current methodology of complex genetic traits relies largely on high-resolution SNP panels containing up to approximately one million SNPs. The main advantage of GWA studies is that it is hypothesis-free, i.e. no prior knowledge of gene functions is required. In contrast, traditional candidate gene studies are hypothesis-based. Stringent criteria for significance threshold in genome wide studies are necessary since a large number of tests are performed. The current significance threshold for a GWAS is  $P < 5 \times 10^{-8}$ .

GWA studies have also found a large number of common variants for metabolic traits (Ingelsson et al., 2010) whereas by the end of August 2012, only one genome-wide significant ( $P < 5 \times 10^{-8}$ ) variant was characterized for sleep duration. One of the reasons why GWA studies have captured common variants more than rare variants is simply due to the fact that the platforms are designed to contain common variants that are thus better presented in current genotyping platforms. The elucidation of rare genetic variants will require larger sequencing efforts, customized GWA study platforms or high-quality imputation of rare markers that are only starting to emerge in the field of genetics.



Since 2005 when the first GWA studies were published, our understanding of the etiology of complex genetic traits has grown significantly. On top of the GWAS platforms, the 1000 Genomes and HapMap Project data enable the assigning of genotypes (imputation) of an additional 8 to 32 million SNPs (The 1000 Genomes Project Consortium, 2010, The international HapMap Consortium, 2005).

Exome sequencing and whole genome sequencing studies have now started to elucidate the role of the rare genetic variants for example in type 2 diabetes in *MTNR1B* (Bonnetfond et al., 2012) but also in the field of sleep research. One study described a variation in the circadian pacemaker gene *DEC2* in familial short sleepers, whereas another found variation in familial narcolepsy with *DNMT1* (He et al., 2009, Winkelmann et al., 2012).

### 2.1.6 Missing Heritability

Even though GWA studies have been successful in finding genetic polymorphisms for several traits, the current findings only explain a fraction of the total heritability of a trait. The unexplained part of heritability by currently known markers is referred to as missing heritability (Maher, 2008). There are several factors that may explain why all of the variants are not discovered. On the first hand, the current GWAS platforms are concentrated on common variation between individuals. It may be that there are rare variants, which explain part of the missing heritability that are not captured by the current methods. These variants are too rare to be captured by GWA studies but do not have sufficiently large effect size to be found with traditional linkage analyses.

On the other hand, heritability estimates do not take into account interaction, epistasis or gene networks. This means that current heritability estimates may overestimate the number of variants that are expected to be found for each trait (Zuk et al., 2012). It has also been shown that genes that form pathways explain a larger part of the heritability when studied as pathways and less of the heritability when studied as single variants alone. Thus, the whole network has a larger effect than the sum of the individual variants alone. Others have suggested that the heritability could reside outside SNPs either in the epigenetic regulation of diseases or in other polymorphisms, such as CNVs or repeat polymorphisms (Hannan, 2010).

### 2.1.7 Challenges in the Analyses of Complex Traits

Several factors can create challenges in analyzing genetic data and create variation either at the genetic or phenotypic level. First, only a few phenotypes can be measured without any bias. Most of the psychiatric traits are measured using questionnaires or diagnosis by a physician, which both creates subjective measurements and may thus bias the phenotype. For some traits, such as sleep

measured with polysomnography, there is a way to measure the trait objectively. However, these measures cannot be done in large scale currently due to time and cost aspects. One solution is to verify the statistically most significant findings in experimental settings, such as in animal models, and characterize their biological role in general in more detail (Dermitzakis, 2012).

Most complex diseases have a variety of symptoms that can vary in severity, making it more challenging to decide whether a person should be defined as a case or a control. Similarly, differences between populations can be substantial. The scale of body mass index (BMI) is different for individuals from Europe and individuals from Asia. For example, the cut off BMI values for overweight are 30 on the Western scale but 27.5 for the Asian scale.

Phenotypes from biologically separate traits can overlap or display as phenocopies. These phenocopies have a different genetic composition, however. On the other hand, individual genotypes may have different effects depending on the environment; different environments produce different phenotypes. Similarly, genotypes may interact together creating different phenotypes. Maybe the most dramatic evidence of such interaction is fatal familial insomnia and Creutzfeldt Jakob disease, where one SNP D178N at the coding region of the PrP gene often causes the disease but the severity of the phenotype and the progression of either disease is dependent on the other SNPs in the gene (Capellari et al., 2011). SNPs can thus have different effects for different diseases. Genetic effects may also be beneficial for one and deleterious for another trait. Such variants are known for example for autoimmune diseases where a variant in *PTPN2* increases the risk of type 1 diabetes and rheumatoid arthritis, but is protective for Crohn's disease (Barrett et al., 2008, Plenge, 2008).

Population stratification can create variation in the analysis. For example, there is relatively large variation between geographical regions in Finland (Jakkula et al., 2008). Adjusting for population stratification based on geographic location and adjusting for genetic principal components largely reduces the variation due to geographic differences (Jakkula et al., 2008, The 1000 Genomes Project Consortium, 2010).

The studied variant only rarely is in complete LD with the causative variant and the used platform may not contain any variants in the causative region. However, haplotype analysis and exome sequencing can overcome some of these challenges by introducing more information on the studied locus. The search for common variants with low effect size or rare variants with high effect size also requires large sample size and sophisticated statistical methods in order to have sufficient power to detect the associating variants (Liu and Leal, 2010).

Complex traits by definition are affected by more than one gene and it is possible that there is genetic interaction between the markers or between a marker and the environment. The possibility of genetic interaction is normally not taken into

account in heritability estimates, which may eventually lead to overestimation of the amount of variants that contribute to a trait (Zuk et al., 2012). The gene-environment interaction studies as well as gene-gene interaction studies also need large sample sets in order to overcome multiple testing issues (Wang and Zhao, 2003, Smith and Day, 1984).

### 2.1.8 Association Analysis and Biological Function

For several complex genetic traits, such as blood lipid levels or T2DM, many of the common genetic variants are now known (Kettunen et al., 2012, Teslovich et al., 2010). It is, however, even more important to know why some changes at the level of DNA are related to a disease and how the changes in the phenotype are caused. In effect, the next step is to connect the individual variants with biological function. More precisely, what the consequences of having a certain variant are. One possibility is to look for the gene expression of nearby genes in relation to variants that associate with a trait (Cheung et al., 2005, Goring et al., 2007). These expression quantitative trait loci (eQTL) provide the information whether a polymorphism is related to changes in gene expression that might affect the trait. Similarly, effects of SNPs can be studied together with epigenetic changes (Kilpinen et al., 2012).

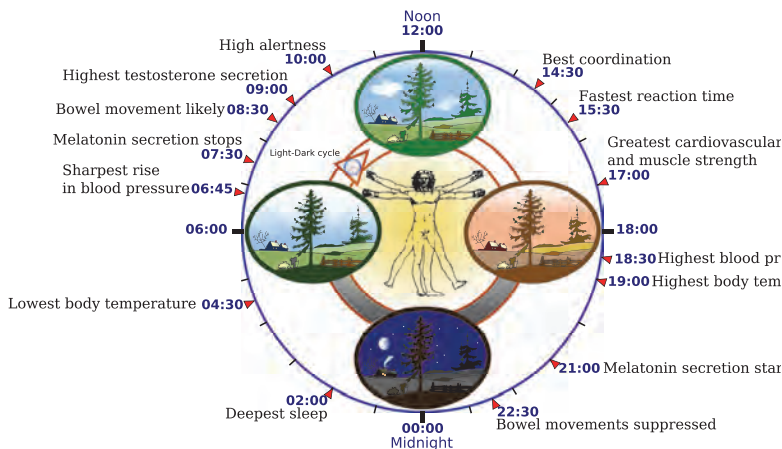
One method is to characterize the effects of associating variants or nearby genes in animal models. This approach has been used successfully in sleep research where a variant in *DEC2* was discovered in a family with short sleep duration. The gene was further characterized in an animal model. The mice deficient for *DEC2* did not show difference in their sleep duration. However, shorter sleep duration was seen only when the same mutation was introduced to the mice (He et al., 2009).

It would always be necessary to relate functional studies back to human physiology. One possibility is to perform studies in relation to human phenotypes in a controlled environment. In the field of sleep research such approaches have been applied in sleep restriction models. These studies have characterized a VNTR in *PER3* that associates with sleep intensity and cognitive performance after sleep loss (Viola et al., 2007, Groeger et al., 2008) as well as with diurnal preference (Archer et al., 2003).

## 2.2 Sleep and Circadian Rhythm

### 2.2.1 What is Sleep?

Sleep is characterized by reduced consciousness, prolonged reaction time, immobility, and declined posture. So far all animals are known to sleep. Circadian processes in turn regulate the timing of sleep, as well as feeding and several other physiological processes such as daily rhythms in temperature, hormonal signals, performance and mood, illustrated in Figure 5. Both sleep and circadian rhythms are tightly regulated. However, it is not known why we sleep even though we spend a substantial amount of time, a third of our lives sleeping. Several hypotheses for the function of sleep exist, however, and many of those agree that sleep is restorative or they link sufficient sleep with conservation of energy or with brain plasticity (Cirelli and Tononi, 2008).



**Figure 5.** The circadian variation in physiology (Wikimedia commons). Mood and body functions show circadian and circannual rhythms. Highest testosterone and alertness levels occur in the morning whereas best physiological performance takes place in the afternoon. Desynchrony in circadian rhythms is related to diseases.

### 2.2.2 Measuring Sleep

In humans sleep is usually measured in sleep clinics with polysomnography (PSG), which comprises of signals from electroencephalogram (EEG), electro-oculography (EOG) and electromyography (EMG). Different sleep stages can then be scored based on their polysomnographic fingerprint. Sleep can also be assessed with movement detectors, actigraphy, or with questionnaires.

### 2.2.2.1 Polysomnography

Human sleep consists of periods of wake (W) and two distinct types of sleep: rapid eye movement (REM) that is often marked in sleep scoring with R and nonREM sleep (NREM) that can be further divided into three stages N1-N3 based on the PSG signals. N1 reflects the lightest and N3 the deepest NREM sleep stage (Iber, 2007). The sleep stages can be characterized based on their PSG profile by interpreting EEG, EMG and EOG signals together. The PSG of REM sleep is characterized by muscle atonia seen in EMG. The characteristics of REM sleep in EEG are similar to waking EEG with both high and low amplitude signals. Most dreams occur also in REM sleep but some can also be present in NREM sleep. NREM sleep has high voltage and low frequency EEG activity without muscle atonia and especially N3 is characterized by deep slow wave sleep (SWS) delta waves that reflect sleep density. NREM and REM sleep occur cyclically during the night so that light sleep is followed by deeper sleep, and finally a REM sleep episode (Rechtschaffen A. 1968).

Usually, the first part of the night in humans is abundant in NREM, especially in SWS sleep, whereas the proportional amount of REM sleep per sleep cycle increases toward the end of the sleep period (Rechtschaffen and Kales, 1968). In addition, the frequency of the EEG waves can be studied. The delta 0.5–4Hz frequencies correspond well with deep stage 3 sleep, slow wave sleep (SWS), and are referred to as slow wave activity (SWA). The other frequencies are theta 4–8Hz, alpha 8–13Hz and beta >13Hz. The delta and theta frequencies have been connected with sleep homeostasis as the SWA delta power can be used as a homeostatic marker for estimating the need for sleep, i.e. sleep pressure (Brunner et al., 1993). Delta waves have also been related to memory processing during sleep, meaning that activity bursts in SWA may strengthen the connections between synapses (Hanlon et al., 2011).

In addition to sleep EEG, the waking EEG can also be used for studying sleep. The waking EEG theta activity can be used as a marker for the build-up of sleepiness (sleep pressure) both in rats and humans (Brunner et al., 1993, Finelli et al., 2000, Vyazovskiy and Tobler, 2005). More generally, theta has been related to attention, memory processing and the plasticity of the brain. A confounding factor in human EEG is age. Human EEG changes over age so that the amounts of SWS, REM sleep, and N1 and N2 sleep decrease with age, whereas the number of awakenings after sleep onset increases (Ohayon et al., 2004).

### 2.2.2.2 Actigraphy and Questionnaires

During sleep cycles individuals show differences in the amount of movement. Sleep and circadian rhythm can be measured with motor activity using a watch-like device called an actigraph. The actigraph detects movement and is thus able to define for example sleep duration, even though differentiating between the sleep stages is not possible based on actigraphy alone. However, sleep time measured with actigraphy shows over 90% correlation with PSG scoring (Ancoli-Israel et al., 2003). Other

studies have shown that sleep wake cycle and sleep duration are well scored with actigraphy alone (over 0.9 correlation with PSG) but that more detailed analysis such as sleep onset or sleep efficiency were hard to measure (Reid and Dawson, 1999). Measurements with actigraphy are significantly cheaper compared to full PSG recordings and can be analyzed automatically. Actigraphs are ideal for research use due to their low cost and the fact that they can be worn for several weeks. In clinical use many sleep disorders can be detected without overnight recordings at the clinic by using actigraphy in the patient or study subject's home. The American Academy of Sleep Medicine does not, however, recommend using actigraphy instead of PSG recordings in diagnosis or in management of sleep disorders as many sleep disorders cannot be diagnosed by movement signal alone. Nonetheless, they suggest that it can be used for assessing specific sleep disorders such as insomnia, excessive sleepiness and circadian rhythm sleep disorders.

An alternative method for measuring sleep is to use questionnaires or single questions that are often included in population cohorts. A number of scales have been developed for measuring different sleep parameters such as sleepiness (Epworth sleepiness scale), insomnia, chronotype (morningness-eveningness questionnaire), circannual rhythms (global seasonality pattern assessment questionnaire) and sleep duration and quality questionnaires like the Pittsburgh sleep quality questionnaire, (Buysse et al., 1989, Horne and Ostberg, 1976, Johns, 1991, Rosenthal, 1984). Questions about sleep are also part of many questionnaires assessing psychiatric diseases such as depression in the Beck depression inventory (Beck et al., 1961). Sleep questionnaires are useful for large-scale studies but may present a subjective bias in the measurement. However, for some sleep disorders the subjective measurements are more relevant than the objective ones. For example in insomnia, patients often report daytime sleepiness and “not being able to sleep during the night” even though PSG findings show sleep during the night. However, time spent in SWS is often reduced in insomniacs (Dorsey and Bootzin, 1997, Edinger et al., 2004). Choosing the correct method for studying sleep is thus important and often a compromise between time and money consuming PSG measurements, compared to relatively inexpensive questionnaire based studies. Combining several methods may also provide a more thorough understanding of sleep and the studied phenotypes.

### 2.2.3 Neuroendocrine Regulation of Sleep and Circadian Rhythms

#### 2.2.3.1 Neurological and Chemical Correlates of Sleep and Wake

Sleep and wake are regulated by the wake-promoting and wake-inhibiting neurotransmitters that together form a flip-flop switch, providing a rapid transition from wake stage to sleep stage. The switch is controlled by wake-promoting and sleep-promoting neurotransmitters and by the circadian input that can reduce the sleep drive reviewed in (Stenberg, 2007). The wake-promoting neurons in the

ascending reticular arousal system (ARAS) promote cortical arousal via two neuronal pathways: the dorsal route through the thalamus, and a ventral route through the hypothalamus and basal forebrain, which in addition receives activating inputs from orexinergic and melanin concentrating hormone (MCH) containing neurons. In more detail, the ARAS contains the noradrenergic neurons in the ventrolateral medulla and locus coeruleus, cholinergic neurons in the laterodorsal tegmental nuclei and in the pedunculopontine nuclei (Hallanger et al., 1987), serotonergic neurons in the raphe nuclei, dopaminergic neurons in the ventral periaqueductal gray matter and histaminergic neurons of the tuberomammillary nucleus. In addition, orexin and MCH neurons from the lateral hypothalamus and GABAergic neurons from the basal forebrain contribute to the arousal signal.

The main sleep-promoting network is located at the ventrolateral preoptic nucleus VLPO, which has neuronal projections with inhibitory GABA and galanin neurotransmitters that project to the nuclei involved in ARAS (Fuller et al., 2006). These inhibitory networks are active during sleep, repressing the ARAS (Pace-Schott and Hobson, 2002, Saper et al., 2001). Normally, transition stages between wake and sleep do not occur. However, occasional unexpected transitions during drowsy driving or napping can take place. Similarly, sleep disorders can arise when some of the flip-flop switch components are defective. An example of this is narcolepsy where the wake-promoting orexinergic signals are not present (Lin et al., 1999, Nishino et al., 2000), leading to increased sleepiness during the day and fragmentation of sleep. Another example is REM sleep behaviour disorder where dreams are acted out (Schenck et al., 1986).

## 2.2.4 Lipid and Carbohydrate Metabolism in the Brain

The metabolic rate in the brain is high as it consumes 20% of total body oxygen, receives 15% of the cardiac output and uses 25% of the glucose (Zauner et al., 2002). The energy demands of the brain are however reduced in sleep by 44% in the cerebral metabolic rate of glucose and 25% in O<sub>2</sub> (Madsen and Vorstrup, 1991, Maquet, 1995). Deviations in energy metabolism can have a large impact on brain functioning as the brain actively regulates the homeostatic energy balance of the body. One function of sleep has also been suggested to be the restoration of the brain's energy stores (Benington and Heller, 1995).

### 2.2.4.1 Sensing Energy Metabolites in the Brain

The major source of energy in the brain is glucose and its levels in the bloodstream are tightly controlled in order to keep blood glucose stable. The brain plays an important role in regulating fatty acid levels and body weight as well. This regulation is done in order to prevent hypoglycaemia as a fall in blood glucose levels could have detrimental effects on brain functioning. The main site in the brain for regulating glucose homeostasis is the hypothalamus, which combines information from the peripheral glucose sensors and circulating leptin and ghrelin levels and

from other brain regions that receive signals from the peripheral tissues. The hypothalamic neurons activate sympathetic and parasympathetic neurons that regulate glucagon and insulin secretion (Thorens, 2011). The brain can detect at least insulin, leptin, glucagon-like peptide 1 and 2, and ghrelin. Besides direct neuronal connections to the pancreas, a negative feedback loop regulates appetite and glucose production that is sensed and modified by insulin signaling in the hypothalamus (Caspi et al., 2007, Schwartz and Porte, 2005). In addition, the central administration of insulin inhibits food intake (Air et al., 2002). Similarly, the brain senses circulating energy carriers like glucose (Burcelin et al., 2000, Pocai et al., 2005) and fatty acids (Obici et al., 2002). The communication is bidirectional as the fat tissue sends feedback hormones to the brain after taking in glucose or fatty acids. These include cytokines like TNF $\alpha$ , IL-1 $\beta$ , IL-6 and leptin, which inhibit food intake (Ahima and Flier, 2000). Interestingly, the same cytokines and leptin are known to increase after sleep restriction (Irwin et al., 2006, Spiegel et al., 2004a, Spiegel et al., 1999, van Leeuwen et al., 2009).

### 2.2.5 Metabolic Control of Sleep

Increase in local neuronal activity during waking increases neuronal energy consumption (Attwell and Laughlin, 2001) and is dependent on the timing, duration and location of the activity (Sokoloff, 1977). Increased neuronal activity is related to higher excitatory glutamatergic neurotransmission, which requires energy (Attwell and Gibb, 2005). Glucose transported via the circulation and subsequently ATP hydrolysis via glycolysis followed by oxidative phosphorylation is the main energy source for the brain to cover increased energy needs. In addition to glucose, lactate has been suggested to function as an energy source for the brain during increased activity (Pellerin et al., 2007, Pellerin and Magistretti, 1994).

#### 2.2.5.1 Adenosine

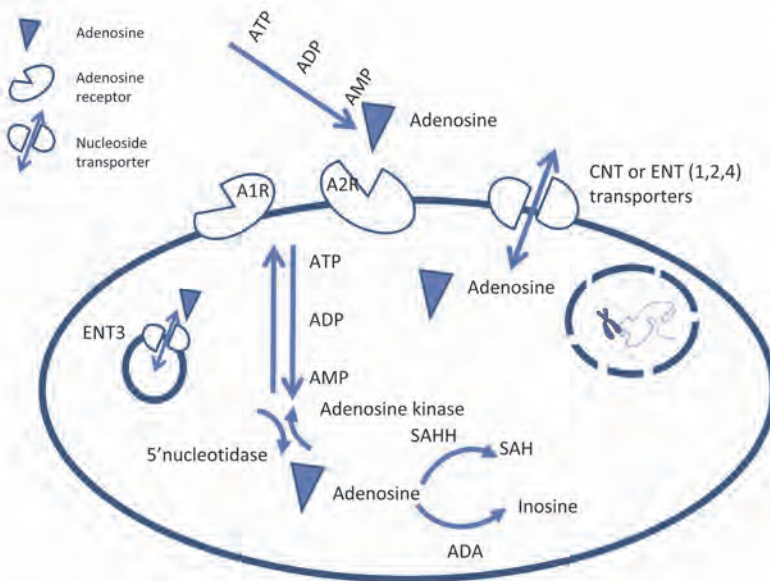
When ATP demands are greater than the production of ATP, adenosine starts to accumulate in the extracellular space. Adenosine is an end product of ATP that can inhibit neuronal firing and induce sleep. It can thus protect neurons from running out of energy and from excessive firing (Dunwiddie and Masino, 2001, Latini and Pedata, 2001, Porkka-Heiskanen et al., 2002). An overview of the genes related to adenosine metabolism is presented in Figure 6. In short, adenosine binds G-protein coupled receptors A1, A2A, A2B and A3. The effects of adenosine on sleep are largely mediated through A1 and A2A receptors and the binding of adenosine to A1 receptors, which inhibits the influx of calcium and glutamatergic signaling (Kocsis et al., 1984, Rebola et al., 2005) and induces the transcriptional activation through NF- $\kappa$ B mediated signaling (Basheer et al., 2001). Cellular and extracellular levels of adenosine are mediated through ENT and CNT transporters, which shuffle adenosine between these compartments and cellular organelles. Another source of



adenosine both inside and outside the cells is synthesis from ATP (Porkka-Heiskanen and Kalinchuk, 2011).

### 2.2.5.2 Hormones

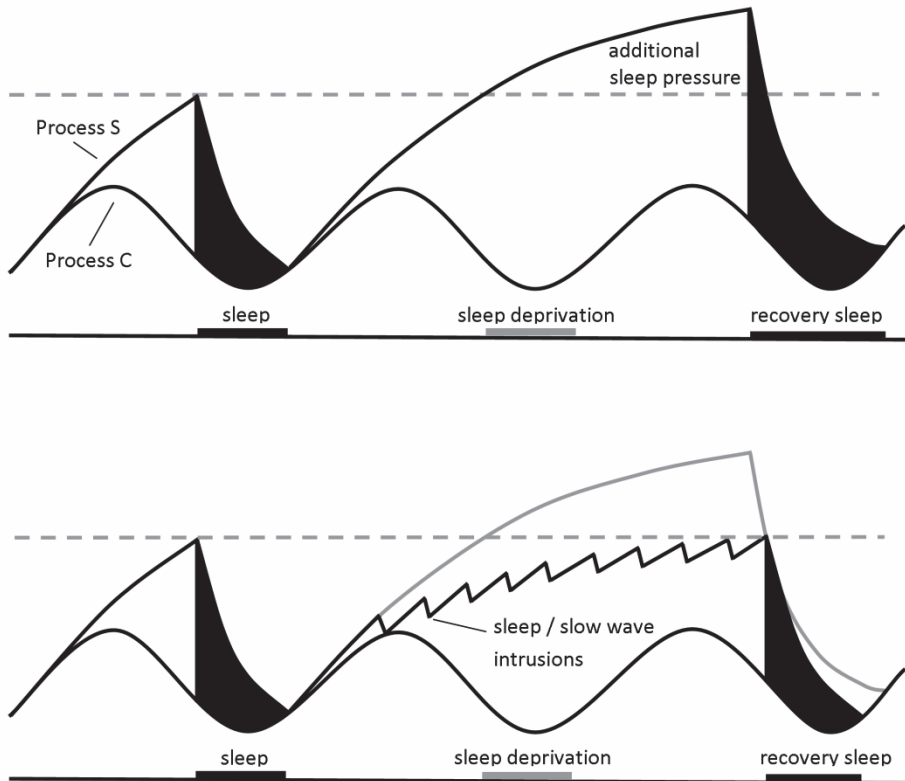
Human and murine EEG studies have been efficacious in studying the connection between sleep and other physiological processes, especially energy metabolism and endocrine functions that are related to changes in vigilance states and in sleep stages. SWS has a role in growth hormone (GH) secretion. GH secretion is controlled by sleep homeostat and increased in the first SWS episode (Van Cauter and Plat, 1996). Sleep also correlates with prolactin secretion. Even naps increase prolactin secretion (Van Cauter and Refetoff, 1985). Interestingly, sleep homeostasis and the duration of SWS also correlate with prolactin secretion (Linkowski et al., 1998). Furthermore, SWS has been shown to inhibit thyroid stimulating hormone and cortisol release (Follenius et al., 1992, Goichot et al., 1992). In addition, NREM sleep decreases heart rate (HR), blood pressure (BP) and vasoconstrictor tone reducing sympathetic nervous activity and increasing parasympathetic activity (Burgess et al., 2004, Somers et al., 1993, Van de Borne et al., 1994).



**Figure 6.** Adenosine metabolism (© Hanna Ollila). Adenosine is the end product of ATP hydrolysis. The ENT and CNT transporters shuffle adenosine between cellular compartments and between extracellular and intra cellular space. SAH hydrolase catalyses the hydrolysis of SAH to adenosine (and back) whereas ADA catalyses irreversible deamination of adenosine to inosine.

### 2.2.6 Homeostatic and Circadian Control of Sleep

Sleep is regulated by two independent but interacting processes. The timing of sleep is regulated by a circadian process (process C) over 24 hours, whereas the intensity and duration of sleep is regulated by a homeostatic process (process S) (Borbely, 1982), shown in Figure 7.



**Figure 7. Homeostatic and circadian rhythm.** Sleep is regulated by a circadian, daily rhythm (depicted in the figure as C), which has a self-sustained synchronous rhythm. The homeostatic component (depicted as S) regulates the response to prior waking, defining the pressure of need to sleep. Modified from Borbely et al., 1982. Figure created by Janneke Zant (<http://urn.fi/URN:ISBN:978-952-10-8412-6>). Reprinted with permission.

The circadian process C is not dependent of prior waking but it oscillates within 24 hours creating a circadian (daily) rhythm. It is generated by the master circadian clock that is located in the suprachiasmatic nucleus (SCN) in the hypothalamus in mammals. This self-sustained circadian rhythm entrains the cells and internal physiological processes, such as sleep, energy metabolism, temperature rhythm and cell division in the whole body (Tu et al., 2005, Buhr et al., 2010). The circadian

rhythm is synchronized primarily by light even though other synchronizers (zeitgebers) with a milder effect such as food or exercise, affect the rhythm (Foster and Hankins, 2002, Berson et al., 2002, Lax et al., 1998, Lax et al., 1999, Moore et al., 2002). The photic input is detected by specialized retinal ganglion cells containing melanopsin and conveyed via the retinohypothalamic tract to the SCN (Gooley et al., 2001, Provencio et al., 1998). The neurons projecting from SCN to the pituitary axes control the hormonal regulation of the circadian rhythm, whereas the neurons that project to the autonomic nervous system are related to neuronal control (Buijs et al., 2003).

In contrast, the homeostatic control of sleep is largely dependent on the duration of prior waking so that extended wakefulness is followed by extended sleep, called recovery sleep (Borbely, 1982). In recovery sleep, especially the amount of EEG slow waves are increased and SWA can be used as a marker of sleep pressure or sleep intensity (Borbely, 1982, Borbely et al., 1981, Rosenberg et al., 1976). The homeostatic process thus regulates sleep need by recording the need for rest as sleep pressure that increases during waking and decreases during sleep. However, the homeostatic and circadian processes are connected, and in order to stay awake during the day the circadian alertness is increased to oppose the increasing sleep pressure. Similarly, during the night when sleep pressure decreases, the circadian sleep factor increases, and sleep is maintained (Dijk and Czeisler, 1995).

### 2.2.7 Regulation of the Circadian Rhythm

Clock genes are regulators of the circadian rhythm in cells and form a self-sustained molecular oscillator. They are located in the cells of SCN but also in many other tissues, such as the lungs, skeletal muscles, the pancreas and the liver, all of which contain pacemakers (Balsalobre et al., 1998, Sadacca et al., Yamazaki et al., 2000). The pacemaker genes are bHLH transcription factors that form feedback loops with positive (CLOCK, NPAS2, BMAL1, BMAL2) and negative regulators of the clock (CRY1, CRY2, PER1, PER2 and PER3). CLOCK and NPAS2 are capable of dimerizing with BMAL1 or BMAL2 and forming heterodimers. This transcriptional complex activates a number of genes with the E-box binding site such as CRY and PER proteins and DEC1 and DEC2, RORA, PPAR $\alpha$ , RXR $\alpha$  and Rev-erb $\alpha$ . The negative regulators CRY and PER proteins dimerize and are transported back to the nucleus where they repress BMAL and CLOCK transcription and thus regulate their own expression by forming a negative feedback loop, reviewed in (Lee et al., 2001). Cytoplasmic levels of CRY and PER proteins are regulated by Casein kinase I $\epsilon$  that phosphorylates the proteins for degradation (Price et al., 1998). RORA and PPAR $\alpha$  can directly bind and activate BMAL expression whereas Rev-erb $\alpha$  represses the CLOCK-BMAL complex (Liu et al., 2008). In contrast to genes in circadian sleep regulation, the genes regulating sleep homeostasis are largely unknown.

Circadian pacemakers have an important role also outside the brain in timing cellular and metabolic processes. Problems in synchronizing the peripheral tissues to the correct time may be dependent on at least two factors. First, the lower amplitude of an oscillator, such as poor melatonin rhythm, insulin resistance, irregular mealtimes or blindness, can have a weaker effect on keeping the target tissue in correct time. Second, environmental effects that cause desynchrony, such as jet lag or shift work, can desynchronize the central circadian clock in SCN. In addition, genetic differences in the clock genes can have an effect on the peripheral or central oscillators (Bass and Takahashi, 2010).

#### **2.2.7.1 Melatonin**

Melatonin is a hormone that shows high levels of circadian variation. Its levels are high during the night and low during the day as it is quickly degraded after light input (Brainard et al., 2001). It is thus considered a circadian hormone. The secretion of melatonin is under the control of SCN and it is secreted from the pineal gland even though other tissues such as leucocytes, enterocytes and retinal cells are, to a smaller extent, capable of producing melatonin (Lerner et al., 1960). The exact physiological role of melatonin in humans is unknown. In other animals it is the key signal that regulates annual reproductive rhythms as it signals the length of the day (Yoshimura, 2010). The signal transduction of melatonin is mediated by two receptors: G-protein coupled melatonin receptor 1 A (MTNR1A) and B (MTNR1B). The signal transduction ultimately leads to an increase in cyclic GMP and a decrease in cyclic AMP levels in the cytoplasm and the activation of the AKT/GSK3B-pathway (Shieh et al., 2009).

Melatonin has recently been linked with insulin signaling and T2DM, both at the physiological (Nogueira et al., 2011) as well as the genetic level (Ingelsson et al., 2010). Melatonin has been shown to have an inhibitory effect on insulin secretion under normal conditions via the melatonin receptors (Lyssenko et al., 2009, Staiger et al., 2008, Muhlbauer et al., 2012).

#### **2.2.8 Why Do We Sleep?**

One of the major hypotheses suggests that sleep balances brain energy stores, especially in the form of glycogen in the brain that is consumed during waking (Benington and Heller, 1995). This theory is supported by observations in increased end metabolites after sleep deprivation in the brain. In addition, the homeostatic sleep factor adenosine is an end product of ATP metabolism, which links energy expenditure and energy homeostasis directly with sleep homeostasis (Porkka-Heiskanen et al., 1997). Other theories have proposed that being asleep during the night helps to avoid nocturnal animals that could have, once, during human evolution, posed a threat, and vice versa. A third hypothesis links sleep with memory consolidation and synaptic plasticity. In more detail, this theory suggests

that sleep is needed to establish synaptic homeostasis after waking. Sleep deprivation studies have indeed shown that insufficient sleep reduces learning capacity and that sleep is needed for strengthening connections between synapses (Bushey et al., 2011, Hanlon et al., Yoo et al., 2007).

### 2.2.9 Sleep Duration – How Much Sleep Do We Need

Normal sleep duration is between 7 to 8 hours per day as estimated by the Health 2000 study in Finland, with a mean of 7.51 hours (Kronholm et al., 2006) or from the National Health Interview survey from the United States collected on 1990 with a mean of 7.28 hours (USA Census Bureau). There is a strong gender effect on sleep duration, as females sleep 0.23 hours more in average than men (Kronholm et al., 2006). Although objective sleep duration has

Sleep and watchfulness  
both of them,  
when immoderate  
constitute disease.  
Hippocrates

been shown to decrease with age (Ohayon et al., 2004), population-based studies from Finland have shown a U-shaped relationship between self-reported sleep duration and age which may reflect either changes in sleep architecture over life time or co-morbidities that increase with older age and would thus increase sleep duration (Kronholm et al., 2006).

Several factors can have an effect on sleep duration in addition to age and gender. First, sleep homeostasis regulates sleep duration as sleep loss increases sleep duration the following recovery night (Borbely, 1982). Second, the circadian rhythm has an effect on sleep duration (Akerstedt and Gillberg, 1981) and melatonin can both deepen and initiate sleep (Zhdanova and Wurtman, 1997). Third, lower socioeconomic position is associated with shorter sleep duration (Stamatakis et al., 2007). Fourth, psychological and psychosocial factors have a strong effect on sleep duration. Short sleep and sleep disturbances are more common in individuals suffering from major depression (Riemann et al., 2001), and loneliness or stress (Akerstedt et al., 2007, Cacioppo et al., 2002). Fifth, race and ethnicity have an effect on sleep duration. Sometimes ethnic differences may reflect either cultural differences, such as the siesta in the Mediterranean countries, or socioeconomic or genetic differences. Generally, African Americans tend to sleep more than whites or Hispanics (Hanlon and Van Cauter, 2011). Finally, exercise is known to have a positive effect on sleep duration and sleep quality by increasing sleep duration, decreasing sleep latency, decreasing REM sleep, increasing REM onset latency and increasing N2 and SWS (Youngstedt et al., 1997).

From a study collected in Finland in the year 2000, it was estimated that 28% of the population have either shorter (14.5%) or longer (13.5%) sleep duration than the average 7–8 hours per day. It is thought that many individuals with short sleep

duration suffer from sleep debt rather than being natural short sleepers. It is estimated that 18–25% of Finnish individuals live in constant sleep debt. (Kronholm et al., 2006).

### 2.2.10 Sleep Duration and Morbidity

Sleep duration deviating from the normal 7–8 hours a day has been related to mortality (Cappuccio et al., 2010b) and especially to morbidity and mortality from metabolic and cardiovascular diseases (Johansson et al., 2011, Kronholm et al., 2011). During the past three decades the mean sleep duration has decreased in Finland while complaints of sleep problems have increased (Kronholm et al., 2008). Similar findings have been seen in the United States (Knutson et al., 2010, Rowshan Ravan et al., 2010) and Sweden. A study in the United States found a decrease in sleep duration that was independent of ethnical background (Hanlon and Van Cauter, 2011). Especially large changes have been seen among adolescents (Spruyt et al., 2011, Van Cauter and Knutson, 2008). It is estimated that part of the population is living in constant sleep debt. At the same time as sleep duration has decreased, the percentage of individuals having cardiometabolic diseases has increased (Van Cauter and Knutson, 2008, McGuire, 2011).

Epidemiological studies on sleep duration, sleep quality and insomnia have shown that poor sleep and deviating sleep duration are independent risk factors for the development of cardiovascular and metabolic diseases such as T2DM, hypertension, coronary heart disease (CHD) and obesity. Prospective studies have shown that individuals with deviating sleep duration have a higher risk of obesity (Knutson, 2010, Patel et al., 2006). Obesity and overweight are also becoming more and more common among children and adolescents. In the United States, a quarter of the children under five years of age are either obese or have a higher risk of becoming obese (McGuire, 2011). Short sleep duration is an independent risk factor among both children and adults for obesity and overweight (Buxton and Marcelli, 2010, Spruyt et al., 2011). Among children especially the variability of sleep duration or circadian rhythm between weekdays and weekends predisposes to obesity and to higher levels of circulating LDL-C and CRP (Buxton and Marcelli, 2010).

Similar findings have been obtained from cardiovascular risk factors in the Finnish population. A prospective study performed in Finland discovered that deviating sleep duration is an independent risk factor for cardiovascular mortality in women even when adjusting for age, smoking, BMI, hypertension or cholesterol levels (Kronholm et al. 2011). A similar study in United States found also a connection between cardiovascular diseases and deviating sleep duration (Knutson, 2010).

Several epidemiological studies have shown that deviating sleep duration associates with poor glucose tolerance, T2DM and increased insulin resistance after

adjusting for age, gender, BMI, ethnicity and waist circumference. In addition, prospective studies have shown that poor sleep quality and deviating sleep duration are risk factors for developing T2DM (Cappuccio et al., 2010a, Gottlieb et al., 2005). Together these epidemiological studies show that sleep duration and sleep quality are strongly connected with metabolic regulation. However, the mechanisms connecting sleep duration and metabolism are so far only partially understood.

### 2.2.11 Experimental Sleep Deprivation

Sleep deprivation studies have elucidated the connection and mechanisms between sleep duration and cardiometabolic diseases. These studies have consistently found that glucose metabolism changes after sleep deprivation. The overall levels of blood glucose are decreased after sleep deprivation and there is reduced glucose tolerance, or increased insulin resistance both in total sleep deprivation studies and in partial sleep deprivation studies (Spiegel et al., 1999, van Leeuwen et al., 2010). In addition, postprandial glucose and insulin levels are increased in sleep deprivation compared to baseline levels (Wehrens et al., 2010).

Sleep deprived individuals report hunger (Spiegel et al., 2004b, van Leeuwen et al., 2010) and increase in activity of the brain reward system for food (St-Onge et al., 2012). These observations are partially explained by changes in the levels of leptin and ghrelin hormones that modulate satiety and appetite, respectively. Sleep deprivation has been shown to increase the levels of the appetite promoting hormone ghrelin. Some studies have found a decrease in the levels of the satiety promoting hormone leptin, whereas others have found an increase (Spiegel et al., 2004b, van Leeuwen et al., 2010). The decrease may reflect acute changes in the levels of leptin whereas an increase in leptin levels suggests the development of leptin resistance that is also often seen in individuals with high BMI. Some of the changes in leptin and ghrelin levels may also be explained by the increased energy expenditure seen in sleep deprivation (Caron and Stephenson, 2010). Sleep deprivation also increases levels of stress hormones such as serum cortisol (Spiegel et al., 1999) and norepinephrine (Dettoni et al.), suggesting an activation of the sympathetic nervous system. In contrast, others have reported a dampening of the circadian cortisol rhythm after a one-week sleep restriction (Vgontzas et al., 2004). Accordingly, sleep deprivation has been shown to increase heart rate and blood pressure (van Leeuwen et al., 2010) as well as decrease heart rate variability (Dettoni et al., 2012). However, sleep deprivation also increases venous endothelial dysfunction measured by endothelial dependent venodilatation (Dettoni et al., 2012).

The effect of sleep deprivation on lipid metabolism has not yet been fully characterized. One study has reported increased total cholesterol and LDL-C after sleep restriction in post-menopausal women (Kerkhofs et al., 2007). In contrast, a study in rats found evidence for lower total cholesterol levels (TC) after sleep restriction (Everson and Szabo, 2011).

Several studies have found an increase in CRP levels after sleep deprivation. An increase in CRP levels is a marker of inflammation and it has been suggested that changes in low-grade inflammation may explain some of the comorbidities of deviating sleep duration (van Leeuwen et al., 2009). Accordingly, the number of circulating blood leucocytes increases after sleep deprivation. The same study also found an increase specifically in monocytes and neutrophils and a trend in decreased lymphocyte levels after three nights of partial sleep deprivation (Kerkhofs et al., 2007). Another partial sleep deprivation study found that B lymphocytes are increased and Natural Killer cells (NK-cells) are decreased whereas the total amount of T cells is not changed. The same study has also elucidated leukocyte activation after sleep deprivation so that after stimulation they are more prone to secrete the inflammatory cytokines TNF $\alpha$ , IL-1b, IL6 and IL17 (van Leeuwen et al., 2009). Similarly, acute sleep deprivation increases the proinflammatory markers IL1b, IL6 and TNF $\alpha$  (Irwin et al., 2006), suggesting a possible mechanism for inflammatory activation after sleep deprivation.

Finally, sleep deprivation has a strong effect on mental health and performance. Sleep deprivation increases reaction time and daytime sleepiness, and decreases cognitive performance and mood (Van Dongen et al., 2003, Van Dongen and Dinges, 2003, Dinges et al., 1997).

The recovery of sleep deprivation depends on the magnitude of the restriction (homeostasis). The changes observed after one night of total sleep deprivation usually recover during the first recovery night whereas partial sleep deprivation studies need at least two nights of recovery. For example, heart rate did not recover after two nights of recovery (van Leeuwen et al., 2010) but was even increased after the recovery period even though changes in glucose metabolism recovered (van Leeuwen et al., 2009, van Leeuwen et al., 2010).

A few studies have also investigated the role of sleep extension on physiology. Unlike in the epidemiological studies, so far these studies have not found harmful effects of prolonged sleep on physiology, suggesting that the mechanism between longer sleep duration is either comorbid with other diseases after disease onset or that longer exposure to long sleep duration is needed in order to see the disadvantageous effects (Spiegel et al., 2004a).



### 2.2.12 Genetics of Sleep and Sleep Disorders

Sleep duration is a complex genetic trait. The heritability estimates of normal self-reported sleep duration range from 31% to 44% (Partinen et al., 1983, Heath et al., 1990, Watson et al., 2010). Heritability estimates for sleep traits are presented in Table 1. The first GWA study on sleep and circadian traits, performed in 2007 (Gottlieb et al., 2007), identified one genome-wide significant SNP in *PDE4D* with sleepiness. However, only one common genetic variant contributing to normal sleep duration has been characterized. This variant is located in the *ABCC9* gene that encodes for the *K(ATP)* channel (Allebrandt et al., 2011).

**Table 1. Heritability estimates for sleep and mood.** Heritability is defined by the genetic variation in proportion to phenotypic variation. Heritability, estimated from twin and family studies, show substantial heritability for sleep phenotypes and traits that have been associated with sleep duration: Electroencephalography (EEG), bipolar disorder (BD), total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), body mass index (BMI).

Trait	Heritability	Study description	Reference
Sleep length	0.33-0.45	2238MZ, 4545 DZ	Partinen et al., 1983
EEG	0.96	20MZ, 20DZ	De Gennaro et al., 2008
EEG delta	0.76	91MZ, 122DZ	Van Beijsterveldt et al., 1996
EEG theta	0.89	91MZ, 122DZ	Van Beijsterveldt et al., 1996
EEG alpha	0.89	91MZ, 122DZ	Van Beijsterveldt et al., 1996
EEG beta	0.86	91MZ, 122DZ	Van Beijsterveldt et al., 1996
Chronotype	0.5	2863 MZ, 5917 DZ	Koskenvuo et al., 2007
Epworth sleepiness scale	0.38	818MZ, 742 DZ	Carmelli D et al., 2001
Sleep quality	0.44	2238MZ, 4545 DZ	Partinen et al., 1983
Insomnia	0.28-0.48	1605MZ, 1200DZ 1554 MZ, 2991 DZ	McCarren et al., 1994, Hublin et al., 2011
Depression	women 0.42 men 0.29	Swedish twin sample N= 42161	Kendler et al., 2006
BD	0.73-0.93	Finnish BD family N=723 30 MZ and 37 DZ British twins, Norwegian same sex twins N=303	Edvarsen et al., 2008, Kieseppa et al., 2004, McGuffin et al. 2003,
TC	0.58-0.71	100 MZ, 72DZ, 52 MZ, 89DZ	Knoblauch et al., 1997, Pietilainen et al., 2009
HDL-C	0.61-0.73	100 MZ, 72DZ, 52 MZ, 89DZ	Knoblauch et al., 1997, Pietilainen et al., 2009
LDL-C	0.59-0.79	100 MZ, 72DZ, 52 MZ, 89DZ	Knoblauch et al., 1997, Pietilainen et al., 2009
TG	0.66-0.3-0.4	100 MZ, 72DZ 52 MZ, 89DZ	Knoblauch et al., 1997, Pietilainen et al., 2009
BMI	0.73	Meta-analysis N=140 525 twins	Knoblauch et al., 1997, Elks et al.,2012

### 2.2.12.1 Genetic Findings in Sleep Disorders

The most common sleep-wake disorders are insomnia and sleep apnea. Chronic insomnia is defined by difficulties in sleep onset or in sleep maintenance, early awakenings and not feeling refreshed after waking up. The prevalence of insomnia ranges from 9% to 24% and it is more common in women than in men. Insomnia symptoms increase with age and it is related to higher mortality (Buscemi et al., 2005, Daley et al., 2009) as well as to other comorbidities such as depression, cardiovascular diseases, anxiety and dementia (Hale, 2005). Insomnia may be a result of over activation of the wake-promoting systems. So far, no genome-wide significant findings have been found with insomnia. One GWAS has been published and the most significant findings were located in the receptor tyrosine kinase-like orphan receptor 1 (*ROR1*) and phospholipase C, beta 1 (*PLCB1*) (Ban et al., 2011). However, these findings did not reach genome-wide significance. The genome-wide significant findings with sleep and sleep disorders are presented in Table 2.

**Table 2.** Genetic findings with sleep and sleep disorders from GWA and sequencing studies.

Trait	Gene	Leading variant	Reference
Sleep duration	<i>ABCC9</i>	rs11046205	Allebrandt et al., 2011
Sleepiness	<i>PDE4D</i>	rs1823068	Gottlieb et al., 2007
Narcolepsy	<i>HLA</i>	HLA-DQ(B1/A1)	Matsuki et al., 1992
Narcolepsy	<i>TCRa</i>	rs1154155	Hallmayer et al., 2009
Narcolepsy	<i>CPT1B, CHKB</i>	rs5770917	Miyagawa et al., 2008
Narcolepsy	<i>P2RY11</i>	rs2305795	Kornum et al., 2011
Familial short sleep	<i>DEC2</i>	P385R	He et al., 2009
Narcolepsy	<i>DNMT1</i>	V606F/A570V/G605A	Winkelmann et al., 2012
Restless legs syndrome	<i>MEIS1</i>	rs6747972	Winkelmann et al., 2007
Restless legs syndrome	<i>BTBD9</i>	rs9296249	Winkelmann et al., 2007
Restless legs syndrome	<i>MAP2K5-LBXCOR1</i>	rs1026732	Winkelmann et al., 2007
Restless legs syndrome	<i>PTPRD</i>	rs4626664	Schormair et al., 2008
Restless legs syndrome	<i>TOX3</i>	rs3104767	Winkelmann et al., 2011
Restless legs syndrome	<i>NOS1</i>	rs7977109	Winkelmann et al., 2008

Sleep apnea is characterized by discontinuous and reduced breathing during sleep. The severity of the apnea is based on the number of apnea (total loss of airflow) and hypopnea (partial loss of airflow) events per hour, or the apnea-hypopnea index. Sleep apnea is relatively common with prevalence estimates

ranging up to 28% with mild sleep apnea, and up to 14% with moderate sleep apnea. Sleep apnea is more common and more severe in men than in women and it is related to cardiovascular risk factors, T2DM, hypertension and poor quality of life (Young et al., 2002). No genome-wide significant findings have been published on sleep apnea.

The most common sleep related movement disorder is restless legs syndrome (RLS). It is characterized by the need to move the feet or arms during sleep. Individuals with RLS also often suffer from interrupted sleep or difficulties in falling asleep. RLS affects 10.6% of the population (Winkelman et al., 2006) even though larger prevalence estimates up to 24% have been reported (Garcia-Borreguero et al., 2006). The prevalence of RLS increases with age and it is related to other comorbidities like increased daytime sleepiness, depression, anxiety and cardiovascular diseases (Winkelman et al., 2006). The causes of RLS are not fully understood but iron metabolism is related to RLS, as secondary RLS can be caused by iron deficiency (Allen and Earley, 2007). Patients with RLS benefit from dopamine agonists while individuals with Parkinson's disease often suffer from RLS, suggesting that dopamine signaling may have a role in RLS (Montplaisir et al., 1986).

Unlike in most other sleep disorders, a few specific loci contributing to the genetic aetiology of RLS have been characterized. These genome-wide significant findings are located in *BTBD9*, *MEIS1*, *PTPRD*, and three intergenic regions between *MAP2K5* and *SKOR1*, *TOX3* and non-coding RNA *BC034767*, and in chromosome 2p14. The potential target genes included *MEIS1* as well as *ETAA1* in this region (Schormair et al., 2008, Winkelmann et al., 2011, Winkelmann et al., 2008, Winkelmann et al., 2007). Functional studies on *BTBD9* and *MEIS1* knockout animals have supported their role in the etiology of RLS. *BTBD9* deficient animals show sleep and motor function disturbances as well as elevated iron levels in serum (Deandrade et al., 2012). Similarly, *MEIS1* deficient *c. elegans* show increased ferritin levels (Catoire et al., 2011).

Hypersomnias are characterized by increased daytime sleepiness, difficulties in maintaining wakefulness or increased sleep need. The most common hypersomnias include narcolepsy and idiopathic hypersomnia. Narcolepsy is characterized by increased sleep duration, episodes of cataplexy (loss of muscle tone) that is often triggered by positive emotions. The human leukocyte antigen (HLA) allele *HLA DQB1\*0602* has been strongly associated with narcolepsy (Mignot et al., 2001). Individuals with narcolepsy have low levels or absent hypocretin (orexin) in the cerebrospinal fluid (Nishino et al., 2000). It is thought that narcolepsy is an autoimmune disease that is caused by the destruction of hypocretin neurons in the hypothalamus. GWAS findings have supported this hypothesis, as most of the variants with narcolepsy are located in immunological genes (Hallmayer et al., 2009, Kornum et al., 2011).

### ***2.2.12.2 Genetic Findings in Circadian Pace Maker Genes and Melatonin Receptors in Metabolic Diseases***

The connection between sleep disorders or sleep with cardiovascular and other metabolic diseases seems to be bidirectional also at the genetic level. Genome-wide association studies on blood glucose levels and T2DM have found common variants in clock genes, such as *CRY2* and in melatonin receptor gene *MTNR1B* (Ingelsson et al., 2010) and more recently also rare variants in *MTNR1B* (Bonnetfond et al.). Candidate gene studies of clock genes on metabolic traits have found associations with *BMAL1*, *PER2* and *NPAS2* with T2DM and hypertension (Englund et al., 2009, Monteleone et al., 2008, Woon et al., 2007).

## 2.3 Sleep in Psychiatric Diseases



Nightmare by Henry Fuseli (1741–1825)

Sleep disturbances are frequent in psychiatric diseases. The connection between sleep and psychiatric diseases is clearly bidirectional. Sleep disturbances predispose to psychiatric diseases and psychiatric diseases change sleep architecture or cause sleep disturbances (Borbely et al., 1984, Paunio et al., 2009). The connection of sleep with psychiatric diseases is best characterized by depression.

Ninety percent of individuals suffering from depression have sleep difficulties (Kupfer, 1984). The most common sleep problems are insomnia and hypersomnia. Sleep problems are thus common in major depression. In addition, 40% of individuals who are diagnosed with insomnia have a psychiatric disorder and 46.4% of individuals with hypersomnia have psychiatric disorders (Ford and Kamerow, 1989). The relationship between sleep and depression is thus bidirectional as individuals with major depressive disorder report sleep problems and individuals with sleep problems report negative mood or depression (Ryan et al., 1987). These findings suggest that sleep disturbances and depression are (1) either comorbid or that (2) one is a risk factor for the other. Longitudinal studies have supported the latter. A study performed in twins in Finland found that poor sleep was an independent risk factor for developing life dissatisfaction after a six-year follow-up (Paunio et al., 2009). Other prospective studies have found similar effects with insomnia on depression and other psychiatric disorders (Breslau et al., 1996, Weissman et al., 1997). These studies suggest that sleep problems may be the first signs of a developing depression. On the other hand, sleep problems may be independent risk factors.

In depression and in other psychiatric diseases the characteristics of EEG change. Patients suffering from depression have increased amounts of REM sleep, shorter REM sleep latency, decreased amounts of SWS and increased amounts of light NREM sleep N1 and more fragmented sleep (Borbely et al., 1984). Quite strikingly, individuals suffering from depression have a short-time benefit from total sleep deprivation that is reversed with recovery sleep (Wu and Bunney, 1990). This effect seems to be mediated through REM sleep deprivation as REM sleep deprivation alone, but not NREM sleep deprivation, can elevate mood (Vogel et al., 1980). Individuals with depression are also more prone to suffer from jet lag or shift work, suggesting that the circadian system affects the etiology of depression. For example,

circadian misalignment and shift work worsen depressive symptoms (Cole et al., 1990, Emens et al., 2009, Scott et al., 1997)

Besides major depressive disorder, seasonal affective disorder (SAD), schizophrenia, anxiety disorders and bipolar disease (BD) are closely related to disturbances in sleep-wake regulation (Hakkarainen et al., 2003, Jones et al., 2005). The early symptoms of BD include sleep problems that are seen both in bipolar depression and at the onset of mania (Jackson et al., 2003). In BD the role of the circadian rhythm has come up repeatedly. BD patients also have shorter sleep need, larger seasonal variation in mood and a less stable, more variable circadian rhythm (Hakkarainen et al., 2003, Jones et al., 2005). The physiological and genetic mechanisms linking sleep disturbances with psychiatric diseases are largely unknown. Two theories suggest that stress may play a role in this connection. The first theory suggests that low levels of BDNF could cause depression. It has been shown that stress reduces BDNF levels in the brain and antidepressant treatment increases the amount of BDNF (Duman and Monteggia, 2006). Another stress-induced pathway suggests the involvement of the hypothalamic-pituitary-adrenal (HPA) axis, which is known to act in stress (Hafez and Hafez, 2004). Accordingly, individuals that suffer from depression have higher plasma cortisol levels (Burke et al., 2005) and higher corticotropin-releasing hormone mRNA levels (Merali et al., 2004), suggesting higher activity of the HPA axis.

As sleep disturbances are seen with all psychiatric diseases, it is likely that there are some common factors contributing to mood disorders and sleep regulation. Recently, this connection has been studied at the genetic level with depression. Two candidate gene studies (Utge et al., 2011, Utge et al., 2010) evaluated the shared genetic background for sleep and depression with sleep disturbance. These studies found evidence for specific genetic variants in glutamatergic (*GRIA3*, *GAD1*, *P2RX7* and *DAOA*), serotonergic (*SLC6A4* and *TPH2*), neural plasticity (*DISC1*, *CREB1* and *BDNF*), and HPA axis genes (*CRHR1*) (Utge et al., 2011) that associated with distinct subtypes of depression with either early morning awakenings or fatigue. These earlier findings suggest that the variants also regulate aspects of sleep in depression. One of these detected variants in *GRIA3* was also found to contribute to normal sleep regulation in healthy individuals (Utge et al., 2011), suggesting a shared genetic regulation but also evidencing that these variants may be important in normal sleep duration.

## 3 Aims of the Study

Both epidemiological and experimental studies have indicated a connection between sleep duration and various detrimental health conditions, including metabolic diseases and mood disturbances, but the links between sleep and these conditions have remained largely unexplored.

The main aim of the present study was to elucidate genetic factors and variants that contribute to regulation of human sleep duration and timing, and to elucidate their connections to metabolism and mood disorders.

Specifically the aims of this thesis were to

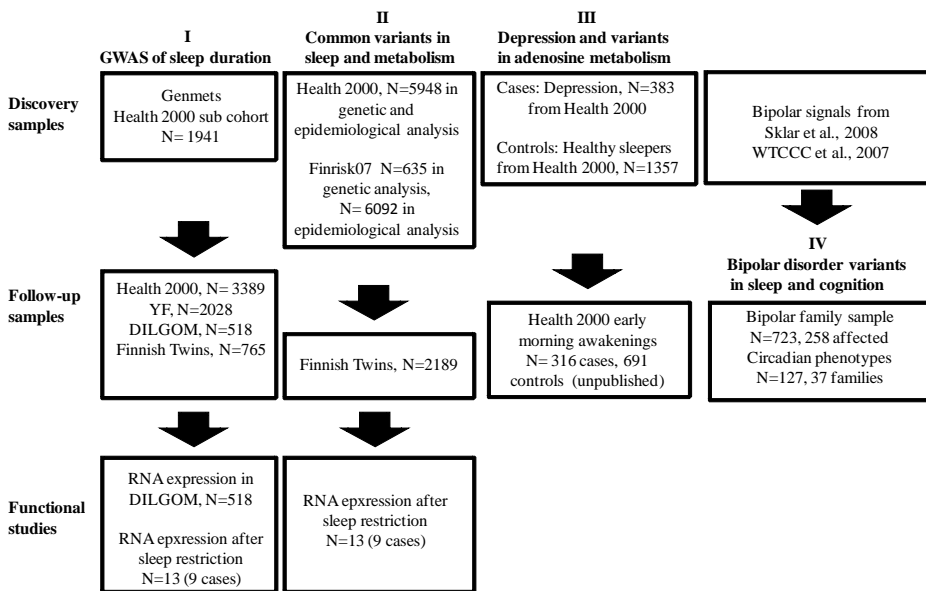
1. Characterize genes and genetic variants that contribute to normal sleep duration (I).
2. Elucidate the cellular mechanisms of short sleep and the effect of sleep deprivation on physiology (I and unpublished).
3. Elucidate the connection of sleep with somatic (II) and psychiatric (III, IV) diseases at the genetic level.



## 4 Materials and Methods

### 4.1 Study Subjects

Figure 8 illustrates the study design for each study. The number of individuals for the cohorts is presented in Table 3. The selection criteria for the individual cohorts and the sub cohorts are described below.



**Figure 8. Study design.** Discovery and follow-up samples are presented. Study I and III include independent discovery and follow-up samples. For study II the markers were selected based on a GWA study of lipid traits published by Teslovich et al., 2010 and studied for sleep duration in the Finnish population-based sample Health 2000 and Finnisk07. For study IV the variants were selected based on published association studies on bipolar disorder (Baum et al., 2008a, Ollila et al 2009 and WTCCC 2007) and it consists of a follow-up of those variants in the Finnish bipolar family sample.

**Table 3.** The number of individuals in each cohort and study.

Subjects	N	Study	Description
Health 2000	6,269	II	Epidemiological sample for studying blood lipid levels and sleep duration. Discovery sample for association analysis of known lipid SNPs with sleep duration
<i>GenMets</i>	1,941	I	Discovery for sleep duration.
<i>Healthy Sleepers</i> <sup>A</sup>	1,357	II, III	Association analysis of sleep duration and known lipid SNPs was studied in healthy individuals (II), control sample for depression (III)
<i>Depression</i> <sup>A</sup>	383	III	Cases sample, depression with disturbed sleep (III)
Finrisk07	7,993	II	Epidemiological sample for studying blood lipid levels and sleep duration. Follow-up sample for association analysis of sleep duration (I) and discovery sample for association analysis of sleep duration and known lipid SNPs (II). (N=652 for genetic variants I, II).
<i>DILGOM RNA expression</i>	518	I	Follow up sample for GWAS variants from GenMets (I).
YF	2,028	I	Follow up sample for GWAS variants from GenMets (I).
Older Finnish Twin cohort	2,189	I, II	Replication sample for II. Follow up sample for GWAS variants from GenMets (I).
Finnish Bipolar Family Sample	723 (258 affected)	IV	Replication sample for GWA-studies in Bipolar disorder. Sample for studying intermediate phenotypes (endophenotypes) for bipolar disorder.
Experimental Sleep Restriction	13 (9 cases)	I, II	Follow up sample for I and II. RNA expression analysis and correlation analysis of variants with EEG slow wave sleep.

<sup>A</sup> The depression group was divided into sub groups based on fatigue (N=194 women, 103 men) or early morning awakenings (N=109 women, 103 men). Overlap in groups “depression with early morning awakenings” and “depression with fatigue” was 95 for women and 58 for men. The Healthy sleepers and Depression sub groups did not overlap as Healthy sleepers were used as controls for the Depression group.

#### 4.1.1 Health 2000

The Health 2000 study (<http://www.terveys2000.fi/doc/methodologyrep.pdf>) is Finnish nationwide survey collected in 2000–2001. The study subjects of the study were selected from the Social Insurance Institution (SII) of Finland (Kela) based on the criteria that they would reflect the main demographic distributions of the Finnish population. The main aims of the survey were to characterize the public health problems in Finland, including areas on physical and mental health and work-related traits. The study consisted of home interview and a health examination including laboratory examination conducted at a local health center. Blood samples for DNA extraction were taken. Altogether Health 2000 comprised of 8,028 individuals (54% females) over 30 years of age of which 6,269 returned the sleep duration questionnaire. The data collection was conducted earlier by the National Institute for Health and Welfare. Selection of sub groups was done for each study separately (I–III).

Studies I and II include 6,269 study subjects belonging to Health 2000 who answered the sleep duration and who had DNA available for analyses. In addition, 1,894 individuals aged 18–29 belonged to Health 2000 sample of Young Adults sub study. They did not answer the questions about sleep and are thus not included in the analyses discussed in this thesis. A written informed consent was obtained from participants. The study was approved by the ethics committee of the Helsinki University Central Hospital.

#### 4.1.2 Health 2000 sub cohorts: GenMets, Healthy sleepers and Depression

##### GenMets

As the discovery sample for genome-wide association analysis of sleep duration (I) we used previously collected GenMets subcohort (N=1,941) belonging to Health 2000. Originally the GenMets sample was collected in order to study metabolic syndrome: half of the individuals belonging to the GenMets study had metabolic syndrome and half of them were age and gender matched controls (Kristiansson et al., 2012). For the metabolic syndrome study also genome-wide chips were done in the GenMets sample (see section 4.3).

Metabolic syndrome was defined according to the International Diabetes Federation (IDF) criteria ([http://www.idf.org/webdata/docs/IDF\\_Meta\\_def\\_final.pdf](http://www.idf.org/webdata/docs/IDF_Meta_def_final.pdf)) in GenMets, i.e., waist circumference  $\geq 94$ cm in males and  $\geq 80$ cm in females. In addition, the subjects had to have two of the following four criteria: 1) blood triacylglycerols  $\geq 1.7$ mmol/l, blood HDL-C in males  $\geq 1.03$ mmol/l and in females  $\geq 1.29$ mmol/l, 2) systolic blood pressure  $\geq 130$ mm/Hg or diastolic blood pressure  $\geq 85$ mm/Hg or 3) medication for treating blood pressure and 4) glucose levels  $\geq 5.6$ mmol/l.

During the Health 2000 data collection the individuals also answered to the question about sleep duration. We thus utilized the ready genotypes and questionnaire based information of the sleep duration for study I. The study was approved by the ethics committee of Helsinki University Central Hospital. Additional genotyping was performed to cover the whole Health 2000 study for the variants that showed point wise association  $P < 5 \times 10^{-5}$  in GenMets.

### Healthy Sleepers

The individuals in the Health 2000 cohort that did not have depression or other sleep complaints (N=610 women and N=525 men) were defined as Healthy sleepers (II, III). These individuals were originally selected in order to get healthy controls for depression cases (III, Utge et al., 2010). For study II these individuals were studied in order to characterize the association of lipid gene SNPs with normal sleep duration without confounding sleep problems. These individuals also served as healthy controls in the depression study III. For these individuals and markers we performed genotyping but phenotypes collected during Health 2000 collection were used.

### Depression

Individuals with major depressive disorder were selected from the population-based Health 2000 cohort for study III and previously published work (III, Utge et al., 2010). The sample comprised 1,423 unrelated individuals (N cases = 258 women, 125 men and Healthy sleepers were used as controls, N controls = 557 women 483 men) with an age range of 30–88 years from 80 regions of Finland. Depression, i.e. major depressive disorder, during past 12 months, was diagnosed according to the Diagnostic and Statistical Manual (DSM-IV) definitions and criteria for psychiatric disorders by using the research version of the Composite International Diagnostic Interview (CIDI) (Pirkola et al., 2005). The controls did not have depression (III). The depression group was divided into sub groups based on phenotypes: fatigue (N=194 women, 103 men) or early morning awakenings (N=109 women, 103 men). Overlap in groups “depression with early morning awakenings” and “depression with fatigue” was 95 for women and 58 for men. The Healthy sleepers and Depression sub groups did not overlap as Healthy sleepers were used as controls for Depression group. For these individuals and markers we performed genotyping but phenotypes collected during Health 2000 collection were used.

#### 4.1.3 Finrisk

The Finrisk studies aim to characterize cardiovascular risk factors in the Finnish population. These studies were done by the National Institute for Health and Welfare. The first study was conducted on 1972, after which cross-sectional studies have been performed every five years (<http://www.ktl.fi/finriski>). The Finrisk07

study discussed in this thesis included 7,993 individuals (53% females) from six areas of Finland: Helsinki and Vantaa, Turku and Loimaa, and the provinces of North Savo, North Karelia, Oulu and Lapland. The study consisted of home interview and a health examination including laboratory examination.

### **DILGOM Sub-study**

The Dietary Lifestyle and Genetic determinants of Obesity and Metabolic Syndrome (DILGOM) study was originally performed as an extension of the Finrisk 2007 study (Inouye et al., 2010). The aim of the whole DILGOM study was to characterize risk factors for metabolic and cardiovascular diseases in the Finnish population both in epidemiological and in genetic level. The individuals were 25-74 years of age and information on their general and mental health was collected. Altogether, 518 individuals (54% females) had both genotypic and phenotypic information on their sleep duration and genome-wide RNA expression. These individuals were included in the follow-up data set for study I in order to study the variants associating with sleep duration in a larger sample of Finnish individuals. DILGOM was also a part of the discovery sample for study II together with the whole Health 2000 sample. A written informed consent was obtained from participants. The study was approved by the ethics committee of the Helsinki University Central Hospital.

In the DILGOM RNA expression sample (I), the RNA expression was measured from 518 individuals belonging to the DILGOM data set (Inouye et al., 2010). For RNA expression analysis the biotinylated cRNA from 518 individuals from the DILGOM cohort were hybridized onto Illumina HumanHT-12 Expression BeadChips (Illumina Inc., San Diego, CA, USA), using standard protocol. For each sample, biotinylated cRNA preparation and hybridization onto BeadChip were done in duplicates (Inouye et al., 2010). In this sample previously made genome-wide chips and previously assessed phenotypes were used in studies I and II.

#### **4.1.4 Older Finnish Twin Cohort**

The Older Finnish Twin Cohort was started in 1975 and follow ups were done in 1981 and 1990 (Kaprio and Koskenvuo, 2002). Sleep duration was assessed in all years by questionnaires and the answers from 1981 were used in the analysis. Altogether 2265 individuals from 762 families participated in the study of which 765 individuals were used in the study I follow-up sample (Kaprio and Koskenvuo, 2002). The study was approved by the ethics committee of the Helsinki University Central Hospital. The twin cohort was used as a follow-up cohort in studies I and II. For study I previously made genotypes were used whereas for study II we genotyped the follow-up markers.

#### 4.1.5 Young Finns

The Cardiovascular Risk in Young Finns Study (YF) is a prospective cohort. It aims to study cardiovascular risk factors in children and adolescents (aged 3, 6, 9 and 18). The study participants were selected randomly (Jylhava et al., 2012). The first collection was conducted in 1980. Sleep duration was assessed on 2007 when the study subjects were 30 to 45 years of age. A written informed consent was obtained from participants and the study was approved by the ethics committee of University Hospital of Turku (Jylhava et al., 2012). For the study I we used previously done genome-wide genotypes and questionnaire-based sleep duration phenotype. Altogether 2028 individuals had both genotype and phenotype information.

#### 4.1.6 Finnish Bipolar Family Sample

The Finnish bipolar family sample (Ekholm, et al., 2002) is comprised of altogether 723 individuals from 180 families of Finnish origin (IV). In the study IV we analyzed the association of suggestive genome-wide significant findings from two earlier publications for BD (Sklar et al., 2008, WTCCC 2007). In the Finnish bipolar family sample we used bipolar disease and any mood disorder as phenotypes. The affected individuals in this data set (N=258) had a variety of psychiatric symptoms: bipolar spectrum disorder (N=173), psychotic disorder (N=212) and other mental disorders (N=45).

In addition, we followed-up the SNPs that associated with BD in the Finnish bipolar family sample and studied their association with endophenotypes. The endophenotypes included circadian and global seasonality phenotypes that were assessed with morningness eveningness questionnaire and seasonality questionnaire (Horne and Ostberg, 1976, Rosenthal, 1984). These phenotypes were characterized from a subset of 127 individuals from 37 families, and neuropsychological phenotypes were characterized from 159 individuals in 65 families. The neuropsychological tests included 22 neuropsychological test variables from the Wechsler Adult Intelligence Scale Revised (WAIS-R), the Wechsler Memory Scale Revised (WMS-R), the California Verbal Learning Test, the Stroop Color and Word Test and the Controlled Oral Word Association Test. The study was approved by the Ministry of Social Affairs and Health and the Ethical Committee of the National Public Health Institute. In the Finnish bipolar family sample the markers were genotyped for study IV.

#### 4.1.7 Experimental Sleep Restriction Study

The sleep restriction study was originally collected in collaboration with the Finnish Institute of Occupational Health to study the effects of insufficient sleep on human physiology (van Leeuwen et al., 2010, van Leeuwen et al., 2009). In short, thirteen healthy men with complete data, aged 19–29 with a mean age of  $23.1 \pm 2.5$  years participated in the study. All had a regular sleep-wake schedule and habitual sleep

duration of 7–9 h. The experiment group (N = 9) spent 8 h in bed for the first two baseline nights, from 11 PM to 7 AM, followed by 5 nights of sleep restriction, where they spent only 4 h in bed from 3 AM to 7 AM. Two recovery nights of 8 h in bed (11 PM to 7 AM) ended the experiment. The control group (N = 4) spent 8 h in bed (11 PM to 7 AM) throughout the experiment. EEG recordings (Embla, Flaga HF, Reykjavik, Iceland), using a sampling rate of 200 Hz with a bandwidth of 0.5–90 Hz and a continuously present investigator monitored that the participants did not sleep or nap during the periods outside those mentioned above. Energy intake was controlled by meals that were standardized and energy-balanced based on the current national recommendations and they were provided at fixed times of the day and consumed by all participants throughout the experiment. The study design was approved by the ethics committee of the Helsinki University Central Hospital and a written informed consent was obtained from the participants. The experiment was conducted at the Brain and Work Research Centre of the Finnish Institute of Occupational Health (van Leeuwen et al., 2009).

In the sleep deprivation study used in study I and II, RNA was collected from blood mononuclear leucocytes in the morning after baseline, restriction and in recovery phases using a Qiagen RNA easy extraction kit (QIAGEN, Hilden, Germany). The RNA expression levels were tested using Affymetrix U133 Plus 2.0 human genome expression arrays (Affymetrix, Santa Clara, CA, USA). We first performed quality control (QC) for the transcripts in the genome-wide RNA expression chips, Affymetrix GeneChip Human Genome U133 Plus 2.0 that were done in the sleep restriction sample. QC of the expression arrays was performed with GeneSpring GX software (Agilent Technologies, Palo Alto, CA, USA) and two individuals with poor array quality were excluded. The GC RMA algorithm was used for normalizing the data. The Affymetrix detection calls were used for filtering criterion and probes flagged ‘present’ or ‘marginal’ in more than 2/3 of the samples were kept in the analysis. Ensemble database probe set definitions (version *homo\_sapiens\_core\_54\_36*, 8.6.2009) were used for annotating the probes to respective genes. 15,101 probes remained in the analysis after QC. The outcomes used in studies I and II included RNA expression analysis of candidate genes in baseline and in sleep restriction. The RNA expression levels were also correlated with the amount of slow wave sleep. In addition, pathway analysis of genes reacting to sleep restriction was performed to study the overall changes in RNA expression after sleep restriction and the effect of sleep restriction on physiology (unpublished results).

## 4.2 Phenotypes

Sleep length was evaluated with a similar question in all cohorts: *“How many hours do you sleep per day?”* and depending on the cohort the individuals could either write down a number (Health 2000 and Finrisk 2007) or circle an answer (The older

Finnish Twin Cohort and YF). Sleep duration showed normal distribution in all studied cohorts. The chronotypes of the individuals using questions 4, 7, 9, 15, 17 and 19 derived from the morningness-eveningness questionnaire (Horne and Ostberg, 1976). In the twin sample, chronotype was assessed by asking *“Try to estimate, whether you are a morning or evening type person”*. The answering options were: *“clearly morning-type”, “a bit morning-type”, “a bit evening-type”, “clearly evening-type”*. We hypothesized that different genetic variants would contribute to eveningness and morningness. The individuals were thus assigned as morning-type, evening-type or neither of the extreme types, and analyzed against the group that did not show clear preference toward either chronotype. The Finnish version of Epworth sleepiness scale was used to evaluate daytime dozing in the Health 2000 study. The questions were: *“How easily you fall asleep when 1) sitting and reading 2) watching television 3) sitting inactive in a public place 4) as a passenger for an hour in a car 5) lying down to rest in the afternoon 6) when talking with someone 7) when the car is stopping at traffic lights?”*. Fatigue was assessed with a question: *“Are you more tired during the day than other people of your age?”*.

In the Finnish bipolar family sample, circadian and global seasonality phenotypes (Horne and Ostberg, 1976, Rosenthal NE, 1984) were characterized from a subset of 127 individuals from 37 families, and neuropsychological phenotypes were characterized from 159 individuals in 65 families. These tests included 22 neuropsychological test variables from the Wechsler Adult Intelligence Scale Revised (WAIS-R), the Wechsler Memory Scale Revised (WMS-R), the California Verbal Learning Test, the Stroop Color and Word Test and the Controlled Oral Word Association Test.

The metabolic phenotypes from circulating blood included total cholesterol (TC), triacylglycerols TG, high-density lipoprotein cholesterol (HDL-C), C-reactive protein (CRP), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose and fasting plasma insulin.

In the Finrisk 07 sample, TC was measured with the CHODPAP-assay, HDL-C with a direct enzymatic assay and TG with the enzymatic GPO assay (Abbott Laboratories, Abbott Park, Illinois, USA). In the Health 2000 sample HDL-C (Roche Diagnostics, Mannheim, Germany). TC and TG were measured using enzymatic assays (Olympus System Reagent, Hamburg, Germany).

### 4.3 Genotyping and Quality Control

Genotyping was performed with Illumina whole genome chips (Illumina Inc. San Diego, CA, USA) in the Wellcome Trust Sanger Institute, Cambridge. Health 2000 sub study GenMets was genotyped with the Illumina 610 K (Kristiansson et al., 2012). Individuals from DILGOM were genotyped with the same platform while Illumina 670 K platform had been used for the Older Finnish Twin Cohort and YF



(Inouye et al., 2010, Kaprio 2002, Jylhava et al., 2012). Individual markers for follow-up in study I were selected based on the association in the GenMets sample and further studied in DILGOM, YF, Finnish Twins and rest of the individuals belonging to Health 2000 study. The Illumina 670 K platform covered all SNPs selected for follow up study except for one SNP (rs35496280) which was excluded from the analysis. For genotyping accuracy individuals and markers with missing data over 5% were removed from the analysis in the GWA. Gender check, heterozygosity and relatedness check were performed and individuals with IBD over 0.2, or discrepancy in gender were removed in all samples with GWA data available. Hardy Weinberg P-value threshold was  $1 \times 10^{-6}$  and markers with  $AF \geq 1\%$  were kept in the GWA analysis.

As genome-wide chips were not available from the whole Health 2000 study (except for GenMets sub cohort) the variants chosen for follow-up in I were genotyped in that sample with Sequenom Mass array technology (Sequenom Inc. San Diego, CA, USA), based on single base extension and iPLEX Gold chemistry in FIMM Technology Center, Biomedicum, Helsinki. This method was also used studies II-IV with the exception of the genotypes for the 635 individuals in study II and for the follow-up of SNP rs12256138 in the GenMets sample (III) that were obtained from the Illumina 670 and 610 platforms, respectively.

In the QC for the candidate SNPs individuals and markers with missing data over 5% were removed from the analysis. Hardy Weinberg P-value threshold was 0.001. Markers with  $AF \geq 1\%$  were kept in the analysis.

## 4.4 Statistical Analyses

### 4.4.1 Epidemiological Analysis

We studied the association of sleep duration with blood lipid levels (II). The association was tested in Finrisk07 (N=6,092) and in the Health 2000 (5,948) sample using the general linear model (GLM). The analyses were performed using PASW Statistics 18 and all analyses were adjusted for relevant covariates (age, gender, lipid medication, hypnotics, BMI and cohort).

### 4.4.2 Association Analysis and Haplotype Analysis

Sleep duration showed normal distribution in the population level in Health 2000 and Finrisk07 samples. For the GWA study of sleep duration in GenMets and for analyses in the follow-up samples YF, DILGOM, Finnish Twins and rest of the Health 2000 cohort (I), the phenotype (sleep duration) was standardized in each sample by using the standard-beta option provided by PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) (Purcell et al., 2007). Linear regression analysis was used to perform the association analysis and the analysis

was adjusted for age, gender, BMI as well as for genomic principal components (GenMets (Kristiansson et al., 2012), DILGOM, YF and the Finnish Twin sample) and metabolic disorder status (GenMets sample). Adjusting the genetic association analyses by the genomic principal components is a method used to correct for population stratification. Fixed effects meta-analysis was performed for calculating P-values using PLINK. Analysis for gene environment interaction was performed using option gxe provided by PLINK (I, III).

Haplotype analysis (I) was performed using Haploview (Barrett et al., 2005) and phasing was performed using Beagle (Browning and Browning, 2011). This software does phasing based on the Hidden Markov Model for haplotypes.

Pathway analysis for SNPs was performed using SNP ratio test (O'Dushlaine et al., 2009). This program calculates the empirical P-values for individual pathways based on permutation tests. In short, the phenotype is permuted one hundred times and then association analysis is done using the permuted phenotypes. The original association results are then annotated into KEGG pathways and compared to the permuted results that are also annotated into KEGG pathways.

In order to study the power of the GenMets sample in GWA for sleep duration (I), we performed genetic power calculation using the Genetic Power Calculator (Purcell et al., 2003). The power was calculated for an additive model assuming quantitative trait locus (QTL) variance of 1.5%, 5% minor allele frequency and perfect linkage disequilibrium between QTL and the marker.

For the candidate gene studies in II and III in the population data, linear or logistic regression analysis with additive model and with relevant covariates (age, gender and BMI) or chi-square test (III) was performed using the PLINK software (<http://pngu.mgh.harvard.edu/~purcell/plink/>) (Purcell et al., 2007). Sleep duration was normally distributed (II). Other quantitative traits (TG, TC, HDL-C, LDL-C and Epworth sleepiness scale) were squared in order to obtain normal distribution (II). For case control analysis of the dichotomous traits (depression and sleep disturbances), normalization was not done (III). Bonferroni correction was used in order to adjust for multiple testing (II, III).

For family-based analysis of the candidate SNPs (IV), we used fbat for dichotomous phenotypes ([www.biostat.harvard.edu/~fbat](http://www.biostat.harvard.edu/~fbat)) (Horvath et al., 2004) and QTDT for quantitative phenotypes (Abecasis et al., 2000). To complement the QTDT test, Plink was used to analyze the direction of association for quantitative traits using option qfam-total. Age, gender and disease status were used as covariates in the analyses of the quantitative traits (IV). No normalization or correction for multiple testing was performed in study IV.

The softwares summarized in Table 4 were used to analyze the genetic markers and RNA expression.

#### 4.4.2.1 Analysis of RNA Expression

The significance at the single transcript level was calculated using 2-way ANOVA, using control and case groups as one axis and time point as another axis (baseline, deprivation and recovery) using (R/Bioconductor open software packages; <http://www.r-project.org>) (II). In addition, changes from baseline to deprivation were calculated with the Student's T-test within the case groups.

In order to analyze enriched pathways in the RNA expression level (unpublished results) an in-house developed pathway analysis program, CIGA, was used (Pietilainen et al., 2008). Often individual transcripts that belong to the same biological pathway do not reach statistical significance alone. Thus we used an in-house developed pathway analysis program for RNA expression that calculates a cumulative P-value for those transcripts that belong to the same GO and permuted the significant pathways with 1,000 in order to adjust for multiple testing. The 2-way ANOVA was calculated using control and cases groups as one axis and time point as another axis (baseline, deprivation and recovery). The genes were ranked for the pathway analysis program by sorting them based on the significance from 2-way ANOVA. The pathway analysis program calculates the P-value by answering the question: "how likely is it to see this many genes (k) that belong to the studied pathway this high-up in the ranked list of genes (j), when there are altogether t genes that belong to the pathway amongst n genes in the experiment".

$$p(j, k, t, n) = 1 - \sum_{c=0}^{k-1} \frac{\binom{j}{c} \binom{n-j}{t-c}}{\binom{n}{t}}$$

The pathway analysis programs rely on the correct annotation of the genes into the pathways. Thus, it is often the case that different analysis methods and annotations find different pathways for a distinct data set. In order to control for annotation problems we verified the detected pathways with two independent programs IPA (Ingenuity Systems®, [www.ingenuity.com](http://www.ingenuity.com)) and Anduril (Ovaska et al., 2010).

For analysis of RNA expression levels in DILGOM population sample, linear regression was used with relevant covariates (age, gender and principal components correcting for population stratification). The analysis was performed using R version 2.14.2, <http://www.R-project.org/>.

#### 4.4.2.2 Correction for Multiple Testing

Genetic studies produce a large amount of tests that create false positive results if only nominal P-values are considered. In the genome-wide setting (I) we used  $P < 10^{-8}$  as significance threshold and assigned the empirical P-value by permutation. In studies II and III, Bonferroni correction was used in order to adjust for multiple

testing (Dunn, 1961). Study IV was performed as follow-up for previous GWA studies and association analysis (Baum et al., 2008a, Baum et al., 2008b, Ferreira et al., 2008, Sklar et al., 2008, WTCCC, 2007). No correction for multiple testing was done in study IV.

**Table 4.** Summary of the software used in the analysis of the data

Analysis	Software	Reference	Study
<b>Association analysis</b>	PLINK	Purcell et al., 2007	I-IV
<b>QTD</b>	fbat	Horvath et al., 2004	IV
<b>Haplotype analysis</b>	Haploview	Barrett et al., 2005	I
<b>Phasing</b>	Beagle	Browning et al., 2011	I
<b>Pathway analysis for SNPs</b>	SNP ratio test	O'Dushlaine et al., 2011	I
<b>Power calculations</b>	Genetic power calculator	Purcell et al., 2003	I
<b>Pathway analysis for RNA expression</b>	CIGA	Pietilainen et al., 2008	I
<b>Epidemiological analyses</b>	SPSS	IBM	II
<b>General linear model</b>			

# 5 Results and Discussion

## 5.1 Genetic Findings in Normal Sleep Duration (I)

Sleep duration is a heritable trait (Partinen et al., 1983) but only one common variant in *ABCC9* by the time of writing this thesis in August 2012 is known to associate with normal sleep duration in humans (Allebrandt et al., 2011). We aimed to characterize common genetic variants at a single SNP level that associate with sleep length by studying a Finnish population-based sample (N=1,941). We then wanted to elucidate the genetic pathways that are associated with sleep duration by using pathway analysis. The 32 variants showing suggestive evidence for association in the discovery sample were followed up in a sample comprising altogether 6,834 individuals from four Finnish cohorts. The three variants showing nominal association of  $P < 0.05$  in the follow-up sample were studied further in order to gain knowledge of their functional role in sleep regulation with RNA expression analysis on sleep duration using a population-based sample and with RNA expression analysis in an experimental sleep restriction sample of healthy men that was performed in controlled laboratory conditions.

In order to gain information of the power to detect genome-wide significant association ( $P < 5 \cdot 10^{-8}$ ) in the discovery sample, we performed power calculations using an additive model for genetic variants. These calculations were based on the assumption that the associating variant would explain 1.5% of the total trait variation and allele frequency over 5% in the sample of 1,941 individuals. Perfect LD between the marker and the causative variant was also assumed. The calculations evidenced that we had limited power to detect variants with genome-wide significance with only 49% power.

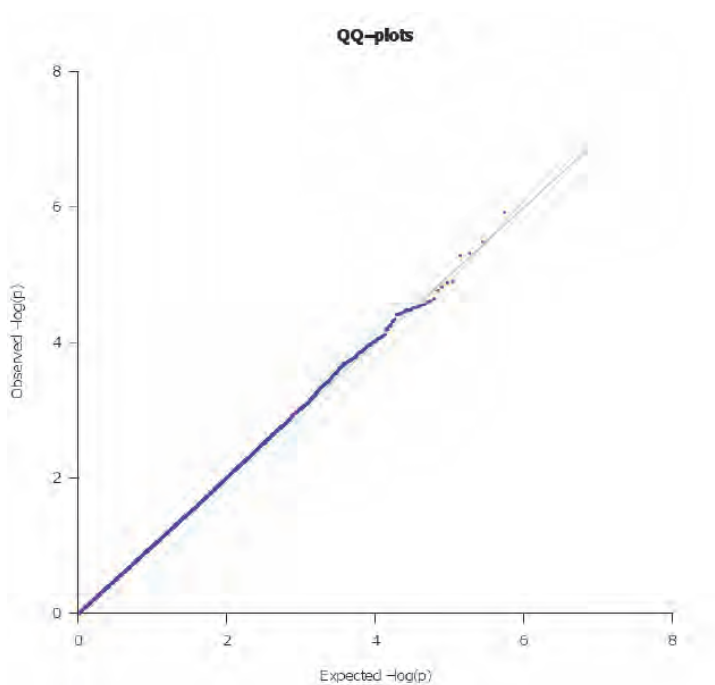
We then performed genome-wide association analysis on sleep duration. In order to visualize the findings, a quantile-quantile (QQ)-plot of the  $-\log_{10}$  P-value distribution (observed P-values vs. expected P-values) was plotted (Figure 9). We observed no deviation from the expected P-value distribution, suggesting that none of the findings were more significant than what would be expected by chance. Similarly, none of the lowest P-values were significant at the genome-wide level as all SNPs had P-values larger than the genome-wide significance threshold of  $P < 5 \cdot 10^{-8}$ . The SNPs and their suggestive association with sleep duration with P-values up to  $5 \cdot 10^{-5}$  are shown in Table 5.

Previous GWA studies have shown that also variants reaching only suggestive levels of significance may be biologically relevant (Lango Allen et al., 2010). A number of interesting candidate genes were found in vicinity of the variants that associated tentatively to sleep duration in GWAS, including glutamate receptors (*GRID1* and *GRI1*), protein tyrosine phosphatases (*PTPRU* and *PTPRT*) and

*KLF6*. Genes from the glutamatergic system, such as *GRIA3*, have been shown to associate with sleep duration in candidate gene studies (Utge et al., 2011) and GWA studies with sleep disorders such as restless legs syndrome have found significant associations with variants in *PTPRD*, another member of the protein tyrosine phosphatase family (Schormair et al., 2008). Moreover, *KLF6* is an activator of iNOS signaling, which regulates sleep homeostasis (Warke et al., 2003). The variants showing suggestive evidence for association (i.e.  $P < 5 \times 10^{-8}$  x  $< 5 \times 10^{-5}$ ) were thus selected for follow-up in larger sample of Finnish individuals (N=6,834) to further study their association with sleep duration.

Altogether three out of 32 SNPs showed association to the same direction as in the discovery sample of point wise  $P < 0.05$  in the follow-up sample. The variants were located near *PTPRU*, *KLF6* and between *CENTD1* and *PCDH7*. The minor allele A of *PTPRU* SNP rs10934351 ( $\beta = 0.037$ , SE=0.019,  $P = 0.049$ , MAF=0.027), and of SNP rs1037079 between *CENTD1* and *PCDH7* associated with longer sleep duration ( $\beta = 0.045$ , SE=0.018,  $P = 0.011$ , MAF=0.10) whereas the minor allele of rs2031573 near *KLF6* associated with shorter sleep duration ( $\beta = -0.037$ , SE=0.019,  $P = 0.044$ , MAF=0.11). These findings may support the role of these genes in sleep regulation. However, as the findings do not sustain correction for multiple testing further association studies on sleep duration are needed to verify the association signals and to detect other variants that contribute to sleep duration.

*PTPRU* belongs to a protein tyrosine kinase receptor family. It is an interesting candidate gene since polymorphisms in *PTPRD* have been associated with restless legs syndrome (Schormair et al., 2008). In order to study whether the associating SNP at the vicinity of *PTPRU* is related to the *PTPRU* gene itself, we performed a haplotype analysis. The relatively rare variant rs10914351 (MAF=0.027) tagged a 38kb haplotype in the discovery and in the replication data sets. The haplotype was unique to the minor allele carriers and was not observed in anyone else in the data set (Figure 10). However, the haplotype did not reach the *PTPRU* gene suggesting that the marker rs10914351 tags some other functional elements in that area that may be responsible for the association with sleep duration rather than direct variation in the coding region of the *PTPRU* gene itself.



**Figure 9.** Quantile-Quantile plot from P-values plotted observed vs. expected.

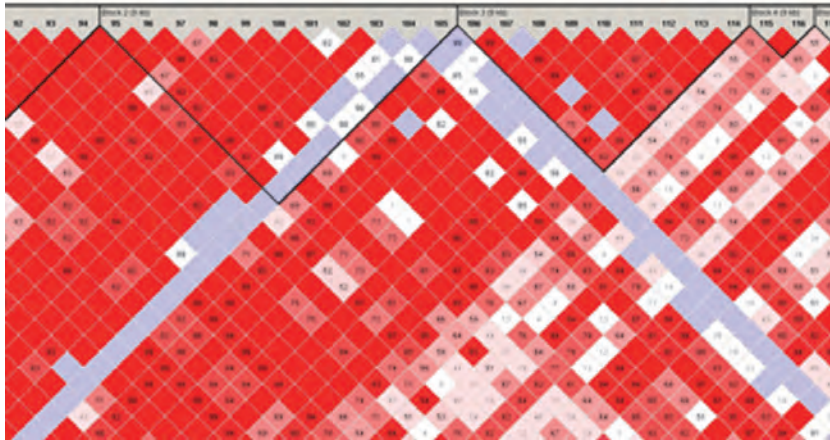
**Table 5. GWA-study of sleep duration.** Original association signals for sleep duration, A1=associating allele, BP=base pair,  $\beta$ =linear regression beta.

CHR	SNP	BP	A1	BETA	SE	L95	U95	P	Gene
9	rs974484	78912590	A	-0.1101	0.02259	-0.1544	-0.06584	1.18E-06	FOXB2-VPS13A
3	rs10934301	116858662	G	0.1054	0.02256	0.06122	0.1496	3.16E-06	GAP43
9	rs4548251	78953578	A	-0.1034	0.02254	-0.1476	-0.05925	4.76E-06	FOXB2-VPS13A
9	rs7869751	78953741	G	-0.1029	0.02253	-0.1471	-0.05876	5.24E-06	FOXB2-VPS13A
1	rs6699898	245329248	A	0.09885	0.02257	0.05463	0.1431	1.25E-05	ZNF669
8	rs4977188	145028452	A	0.09865	0.02256	0.05442	0.1429	1.30E-05	EPPK1
10	rs2031573	3991897	A	-0.0979	0.02254	-0.142	-0.05367	1.49E-05	KLF6
10	rs10886445	121052058	G	0.09723	0.02255	0.05304	0.1414	1.69E-05	GRK5
10	rs12762433	86910260	A	0.09604	0.02261	0.05173	0.1404	2.26E-05	GRID1
10	rs11591606	16951121	A	0.09522	0.0225	0.05113	0.1393	2.42E-05	CUBN
5	rs294704	152499281	C	-0.0949	0.02246	-0.1389	-0.05087	2.50E-05	NMUR2-GRIA1
11	rs2073848	113557114	G	-0.0949	0.02255	-0.1391	-0.05068	2.70E-05	ZBTB16
6	rs9349461	49035104	A	0.0946	0.02249	0.05051	0.1387	2.72E-05	----
10	rs10882611	97284803	A	-0.0954	0.02273	-0.1399	-0.05082	2.84E-05	SORBS1
1	rs10914351	30006618	A	0.09408	0.02247	0.05004	0.1381	2.96E-05	PTPRU
10	rs11188346	97279982	A	-0.0951	0.02272	-0.1396	-0.05053	3.00E-05	SORBS1
10	rs1891787	132798458	A	-0.0943	0.02254	-0.1385	-0.0501	3.01E-05	TCERGIL
6	rs4506041	49083621	A	0.09386	0.0225	0.04976	0.138	3.17E-05	----
14	rs879981	93563662	C	0.09349	0.02244	0.0495	0.1375	3.24E-05	OTUB2
10	rs7072549	86841405	G	0.09405	0.02259	0.04978	0.1383	3.26E-05	FAM190B-GRID1
10	rs10509508	86842628	A	0.09405	0.02259	0.04978	0.1383	3.26E-05	FAM190B-GRID1
4	rs6819546	4902880	G	0.09309	0.02245	0.04908	0.1371	3.53E-05	MSX1
5	rs13185914	152755844	A	-0.0931	0.02246	-0.1371	-0.04904	3.57E-05	GRIA1
17	rs933534	52925956	G	0.09327	0.02253	0.0491	0.1374	3.63E-05	MSI2
1	rs2065954	150587968	C	0.09311	0.02252	0.04898	0.1372	3.70E-05	FLG2
5	rs1429736	117983469	G	0.09303	0.02252	0.04889	0.1372	3.76E-05	----
15	rs4102572	19374161	C	-0.093	0.02253	-0.1372	-0.04884	3.83E-05	POTEB
5	rs1147225	32823941	A	0.09277	0.02248	0.04872	0.1368	3.83E-05	NPRC
4	rs1037079	32233946	A	0.09248	0.0226	0.04819	0.1368	4.45E-05	PCDH7-CENTD1
1	rs6673408	196875928	A	0.0919	0.02249	0.04781	0.136	4.58E-05	PTPRC
1	rs7521839	150651383	G	0.09165	0.02249	0.04756	0.1357	4.80E-05	CRNN
22	rs5746492	16773933	G	-0.0917	0.0225	-0.1358	-0.04755	4.83E-05	MICAL3



**PTPRU haplotype**

A rs10914351-rs12058382  
 B rs995197-rs10914351  
 C rs995197-rs12058382



**Figure 10.** Two common haplotypes from the PTPRU variant rs10914351 were observed: A and B. The minor allele tagged a longer 38kb haplotype ranging from rs995197 to rs12058382 (C) that was not observed in anyone else in the data set.

### 5.1.1 RNA Expression Analysis of Follow-up Variants

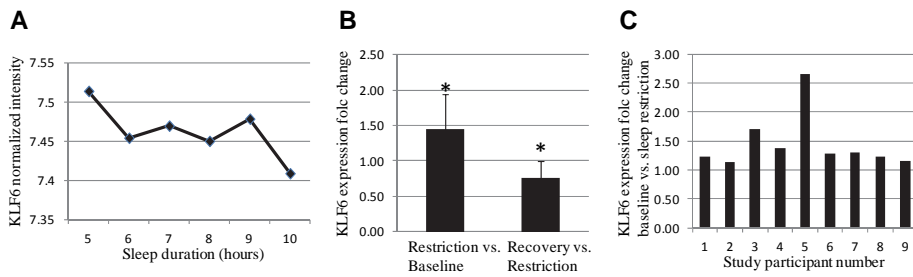
During the time this study was performed, RNA expression data was collected from the DILGOM sample that was part of the follow-up for the GWAS variants. In addition, a collaborative project together with the Institute of Occupational Health was performed to study the effects of sleep restriction on RNA expression levels in blood leucocytes. We thus studied the effect of the three SNPs that showed point wise association in the follow-up sample and their nearest genes (*PTPRU*, *CENTD1*, *PCDH7* and *KLF6*), performing (1) RNA expression analysis of sleep duration in the DILGOM sample and (2) by assessing the change in RNA-expression levels caused by experimental sleep restriction carried out in controlled laboratory conditions. We hypothesized that the expression levels of genes that regulate sleep duration would change if sleep is restricted.

Expression analysis in the DILGOM sample (N=518 with complete data) revealed no eQTLs with rs10914351 and *PTPRU*. The major allele of rs1037079 between *PCDH7-CENTD1* and the minor allele of rs2031573 near *KLF6* that associated with shorter sleep in GWAS, increased the respective gene expression (*PCDH7*/ILMN\_1710544:  $\beta = -0.035$ , SE=0.017 P=0.039, *KLF6*/ILMN\_1700727:  $\beta = 0.040$ , SE= 0.020, P=0.042). Even though these findings were not considered statistically significant as they did not sustain correction for four tests (analysis of illumine probes ILMN\_1657128 and ILMN\_2383300 for *PTPRU* expression,

ILMN\_1709360 for *CENTD1* expression, ILMN\_1710544 for *PCDH7* expression and ILMN\_1700727 for *KLF6* expression from Illumina HumanHT-12 Expression BeadChips), the variants showed a nominal association with the respective gene expression. Higher expression of *KLF6* was also related to shorter sleep length (*KLF6*:  $\beta=-0.59$ ,  $SE=0.36$ ,  $P=0.030$ ) (Figure 11A). These findings suggest that variants rs1037079 and rs2031573 may be eQTLs for their nearest genes. The lack of eQTLs with *PTPRU* may be reflected by the low allele frequency (<5%), and thus a small number of individuals carrying minor allele in the *DILGOM* RNA expression study ( $N=23$ ). The RNA expression study was also performed in blood lymphocytes which may limit the detection of changes in RNA expression since the transcripts may have a brain specific function.

In order to characterize in more detail the role of *KLF6* in regulation of sleep, we studied its expression changes in an independent sample that was not part of the GWAS analysis. In this study sleep was restricted and the study was performed in controlled laboratory conditions in healthy young males in order to minimize confounding factors. We observed that similarly to population RNA expression, the RNA expression of *KLF6* was increased after sleep restriction ( $P=0.006$ ,  $FC=1.4$ , 1555832\_s\_at transcript for *KLF6* from Affymetrix GeneChip Human Genome U133 Plus 2.0), suggesting that shorter sleep duration is sufficient to induce *KLF6* expression (Figure 11B). Despite the relatively large variability of the individual responses after the sleep restriction, we observed that all nine study participants with data available showed increased *KLF6* expression after sleep restriction (Figure 11C).

We hypothesized that the increase in *KLF6* expression might associate with changes in sleep intensity (amount of slow wave sleep), measured by EEG, which is one of the most heritable traits in humans (De Gennaro et al., 2008). Finally we studied the association of *KLF6* expression levels and amounts of slow wave sleep. We observed a positive correlation between *KLF6* expression and the amount of SWS ( $P<0.05$  for 1555832\_s\_at, unpublished observations) further supporting the role of *KLF6* in sleep regulation.



**Figure 11.** *KLF6* in the sleep length groups in DILGOM population sample (A). *KLF6* expression levels after experimental sleep restriction (B) where the sleep restriction values are normalized to baseline and the recovery expression values are compared to sleep restriction. The fold change from experimental sleep restriction comparing baseline to sleep restriction are shown at the individual level (C), baseline = 1.

The findings of the *KLF6* RNA expression study should be considered in the light of following limitations. The original eQTL analysis was performed in the same population sample (DILGOM) that was also used for the follow-up sample for the variants of sleep duration, and the signal for eQTL did not sustain correction for multiple testing. The sleep restriction study was also small, with 9 cases and 4 controls, albeit the number of confounding factors was minimized by careful selection of the study participants for age and regular sleep-wake cycle, with 7 to 9 hours habitual sleep duration. Out of the 54,675 transcripts (for 20,502 genes) that can be measured by the Affymetrix GeneChip, the levels of 4,014 were changed with  $P < 0.05$  after the sleep restriction of 5 nights. According to GeneSpring v.10 the expected number of changes would have been 2733. Similarly, with  $P < 0.01$  we observed 911 changed transcripts whereas 546 transcripts would have been expected to change expression by chance. Thus approximately 40% of the transcripts with similar significance level as *KLF6* ( $P < 0.01$ ) were estimated to be true positive findings. This means that we still had 60% probability of finding a false positive association by chance. However, the *KLF6* levels associated to short sleep both in the DILGOM sample and in the experimental sleep restriction sample. In addition, the findings on association of increased *KLF6* levels with higher amount of SWS, reflecting sleep intensity, further support the association of increased *KLF6* expression levels with shorter sleep duration.

It should also be considered to what extent the changes observed in RNA expression measured from blood leukocytes reflect the changes relevant to sleep. It has also been shown that sleep affects the functions of all tissues of the body that have been tested so far, including blood leukocytes (Irwin et al., 2006, van Leeuwen et al., 2009). In addition, the circadian pacemaker genes are present in all cells of the body, keeping the cells in sync with the suprachiasmatic nucleus (Cuninkova and

Brown, 2008). Sleep has been found to have a strong influence on immune functions since shorter sleep changes the strength of the immune response (Spiegel et al., 2002). Some studies have also shown that immune cells would reflect the expression changes observed in the central nervous tissue to some extent. However, the observations from tissues that are not the direct targets for any phenotype should be interpreted with caution.

To summarize, the consistently most significant findings in study I were observed with one variant near *KLF6*, which encodes a Kruppel-like transcription factor. Increased *KLF6* expression correlated with shorter sleep duration both at the level of population and in an experimental study of sleep restriction. Our findings suggest that *KLF6* expression increases as response to short, potentially insufficient sleep. *KLF6* has been shown to function in cell division and growth. Mutations in *KLF6* in cancers suggest that it has a role as a tumour suppressor, and previous association studies have found that *KLF6* variants associate with prostate cancer. Interestingly, the only study on prostate cancer risk and sleep length showed lower disease risk in those individuals having long sleep lengths of over 9 hours (Kakizaki et al., 2008). *KLF6* is also expressed in the brain: in the cerebral cortex, hippocampus, septum, amygdala, basal ganglia, thalamus, and hypothalamus of mice (Jeong et al., 2009). These findings of *KLF6* on SWS are interesting in light of previous studies showing that *KLF6* has a binding site in the promoter for inducible nitric oxide (iNOS), which can activate iNOS RNA expression and increase the amount of iNOS also in the protein level (Warke et al., 2003). Production of nitric oxide, through activation of iNOS, is increased during sleep deprivation in rats, which has been shown to be a key step in the induction of recovery sleep (Kalinchuk et al., 2006). Our findings are consistent with that *KLF6* may have a role in sleep duration via iNOS mediated action on SWS amount.

### 5.1.2 Pathway Analysis of Sleep Duration

In order to get a broader view of what changes are related to sleep duration, we performed pathway analysis from the GWA study discovery sample. Pathways have been shown to explain more of the genetic variation than the individual GWAS findings in the pathway alone (Lango Allen et al., 2010), which encouraged us to also study the pathways formed by the individual SNPs in sleep duration. We used the SNP ratio test. This program compares the actual association signals with those that are obtained with randomized phenotypes and annotates the overrepresented genes into KEGG pathways. Altogether eight pathways with permuted  $P < 0.05$  were observed. The pathways enriched with sleep duration are presented in Table 6. The results from pathway analysis are always dependent on the correct annotation to the pathways. We thus confirmed that similar association signals were also obtained with another pathway program, Ingenuity Pathway Analysis.

The following pathways were found to be related to sleep duration based on SNP pathway analysis and replicated with Ingenuity pathway analysis (IPA): Natural killer (NK) cell mediated cytotoxicity (in IPA: NK signaling), Inositol metabolism (in IPA: D-myo-inositol-5-phosphate Metabolism) and Long term (synaptic) depression (in IPA: Long term synaptic depression). Genes in these pathways may contribute to normal sleep duration.

The NK cell mediated cytotoxicity pathway most likely presents a pathway that may be related to the pathological states associated with sleep duration, like short sleep or insufficient sleep. It has been previously shown that the amount of NK cells is reduced after sleep restriction, whereas proatherogenic interleukins are increased (van Leeuwen et al., 2009). As part of the population having short sleep duration comprises of natural short sleepers and the other part suffers from insufficient sleep, immunological pathways may be important in regulating the interplay between normal sleep duration and insufficient sleep.

In addition, *GRIA3* was observed in the long-term (synaptic) depression pathway. Variants from *GRIA3* have been previously associated with sleep duration in candidate gene studies (Utge et al., 2011). Even though the pathway ABC transporters were not replicated in IPA, it is interesting to speculate its role in sleep regulation. This pathway included a variant in *ABCC9*, which is so far the only gene that has been found in GWAS on sleep duration (Allebrandt et al., 2011).

It should also be noted that half of the individuals from the discovery sample had metabolic disorder. Such a selection in the discovery sample can bias the findings even though metabolic disorder was included as a covariate in the original analysis. However, distinct pathways, such as long-term (synaptic) depression, suggest a more specific role for the genes in this pathway for sleep regulation. Long term synaptic depression has also been proposed to be the mechanism in sleep-dependent memory consolidation that previously has been proposed to be one of the functions of sleep (Yang et al., 2012).

**Table 6.**

**Pathway analysis of sleep duration.** The pathways from KEGG database (SNP Ratio Test) are shown. P-values reflect the ratio of pathways obtained from association analysis with permuted GWA-studies compared to the original GWAS. The pathways that were also significant (nominal association of  $P < 0.05$ ) with IPA are bolded.

KEGG Pathway ID	P	Pathway name	Genes contributing to pathway
hsa04650	0.016	Natural killer cell mediated cytotoxicity	CD247, FYN, GRB2, IFNA13, IFNA14, IFNA16, IFNA5, IFNA6, ITGB2, KLRC1, KLRK1, KRAS, MICA, MICB, MKRN2, PLCG2, PPP3CA, PRKCA, PTK2B, PTPN11, RAC1 RAF1, SH2D1B, SHC2, SHC4, SOS2, SYK, TNFRSF10A, TNFRSF10B, VAV1, VAV2, VAV3
hsa04010	0.016	MAPK signaling pathway	CACNA1A, CACNA1C, CACNA1D, CACNA1E, CACNA1I, CACNA2D1, CACNA2D3, CACNA2D4, CACNB2, CACNB4, CACNG2, CACNG3, DUSP14, ECSIT, EGFR, FGF1, FGF12, FGF13, FGF14, FGF2, FGF5, FGF6, FGFR2, FGFR4, GNA12, GRB2, HSPB1, IL1R2, KRAS, LRTM2, MAP2K6, MAP4K3, MAP4K4, MAPK10, MAPK8IP2, MAPK9, MKRN2, NTRK1, NTRK2, PAK2, PLA2G12B, PLA2G6, PPP3CA, PRKCA, PRKX, PTPN5, PTPRR, RAC1, RAF1, RASGRF1, RASGRF2, RASGRP1, RASGRP3, RPS6KA2, RPS6KA3, SH2D2A, SOS2, TGFB2, TGFB2, TRAF2, ZAK
<b>hsa00031</b>	<b>0.016</b>	<b>Inositol metabolism</b>	<b>ALDH6A1</b>
hsa05221	0.016	Acute myeloid leukemia	GRB2, KRAS, MKRN2, RAF1, RUNX1, SOS2, TCF7L1, TCF7L2, ZBTB16
hsa02010	0.032	ABC transporters	ABCA10, ABCA4, ABCA6, ABCA7, ABCB5, ABCC1, ABCC9, ABCG1, ABCC6, ABCG2, ABCA12, ABCA5, ABCG8, ABCC8, TAP2, CFTR, ABCA1, ABCA13, ABCC4, ABCC3
hsa03320	0.0396	PPAR signaling pathway	ACSL1, ACSL3, CD36, FADS2, PPARG, SCD, SLC27A6, SORBS1
<b>hsa04730</b>	<b>0.048</b>	<b>Long-term depression</b>	<b>CACNA1A, CRHR1, GNA12, GNAI1, GNAI2, GNAI3, GNAQ, GNAS, GNAZ, GRIA3, GRID2, GRM5, GUCY GUCY1A2, GUCY1A3, GUCY1B3, IGF1, IGF1R, ITPR1, ITPR2, LYN, MKRN2, NPR1, PLA2G12B, PLA2G6, PLCB1, PLCB4, PPP2R2A, PPP2R2B, PPP2R2C, PRKCA, PRKG1, PRKG2, RAF1, RAS, RTDR1</b>
hsa00902	0.048	Monoterpenoid biosynthesis	CYP2C9, CYP2C19

### 5.1.3 Changes in RNA Expression After Sleep Restriction

The pathway analysis from GWAS found a NK cell-signaling pathway connecting sleep duration with immune functions. In population-based samples, one of the challenges in sleep research is to distinguish between normal short sleepers and those that are sleep deprived. There is strong epidemiological evidence between short sleep duration and insufficient sleep with cardiometabolic diseases and mortality (Cappuccio et al., 2010a, Cappuccio et al., 2010b). Sleep restriction and total sleep deprivation studies have found defective glucose metabolism and insulin signaling after sleep restriction as well as increase in inflammatory markers (Spiegel et al., 1999, van Leeuwen et al., 2010), but only one RNA expression study has aimed to elucidate the cellular mechanisms behind these changes in humans (Irwin et al., 2006). To complement our findings on immunological and metabolic pathways in sleep duration, we aimed to characterize the changes induced after sleep restriction in transcriptome in a controlled environment from blood leukocytes in healthy young males. RNA expression at the whole-genome level from three time points was studied: baseline before sleep restriction, deprivation after five nights of sleep restriction and only four hours of sleep per night, and finally a recovery after two nights with eight hours of sleep. This study was performed to reflect the cumulative sleep restriction of a week of extremely hard work.

#### 5.1.3.1 Sleep Restriction

Biological networks were studied using the biological Gene ontology (GO) pathways that were changed after sleep restriction among all transcripts that had passed QC (N=15,101). This was followed by analysis using 2-way ANOVA and ranking the transcripts for the pathway analysis program by sorting them based on significance. Permutation analysis was done by randomizing the transcription values and comparing the original association with the randomized reference in the same data set. In addition, the transcripts were divided into up- or down-regulated based on their expression in the sleep restriction time point to distinguish between activated or inactivated pathways. The findings were verified with two independent pathway analysis programs (Anduril and IPA) in the same data set so that both produced similar top findings as the original association.

The up-regulated GOs comprised of enrichment of inflammation and immunity related GOs ( $P < 0.0001$ ,  $P < 0.005$  after permutation). As expected based on the epidemiological findings, we observed the activation of immunity and leukocyte pathways, and the activation of cytokine pathways, which together comprised the most significant pathways (Table 7). Specifically, the statistically most significant were B cell activation and interleukin-8 production pathways ( $P < 0.1 \times 10^{-4}$ ,  $P$  corrected  $< 0.001$ ). Among the 30 top pathways, genes overlapped considerably (B cell activation, leukocyte activation, cell activation, lymphocyte activation, adaptive immunity, adaptive immune response, adaptive immune response based on somatic

recombination of immune receptors built from immunoglobulin super family domains, leukocyte differentiation, and lymphocyte differentiation). A large part of the up-regulated pathways were related to the function and regulation of the immune system. These included B cell activation pathways. These findings are in line with previous observations from the same data set showing that the amount of B cells is increased in sleep restriction (van Leeuwen et al., 2009). Of the other lymphocyte populations, NK-cells decrease in sleep restriction (van Leeuwen et al., 2009).



**Table 7. Up-regulated pathways after experimental sleep restriction.** The P-values were calculated as point wise P-values and permuted 1,000 times to obtain corrected P-values. The column N genes total shows how many genes are annotated in the pathway. N contributing genes tell how many of the annotated genes are associating in this data set.

ID	Pathway	P	P perm.	N genes total	N contributing genes
GO:0042113	B cell activation	3.62E-06	0.001	90	43
GO:0032637	IL8 production	6.49E-06	0.001	9	8
GO:0001530	lipopolysaccharide binding	6.49E-06	0.001	9	8
GO:0006805	xenobiotic metabolic process	1.23E-05	0.001	8	5
GO:0050817	coagulation	1.40E-05	0.001	75	22
GO:0045321	leukocyte activation	1.77E-05	0.001	208	68
GO:0001775	cell activation	2.20E-05	0.002	217	70
GO:0002460	adaptive immune response based on somatic recombination of immune receptors	2.41E-05	0.002	83	51
GO:0005543	phospholipid binding	2.63E-05	0.003	91	29
GO:0046649	lymphocyte activation	2.96E-05	0.002	184	61
GO:0045416	positive regulation of IL8 biosynthetic process	3.32E-05	0.001	8	7
GO:0042228	IL8 biosynthetic process	3.32E-05	0.001	8	7
GO:0045414	regulation of IL8 biosynthetic process	3.32E-05	0.001	8	7
GO:0015671	oxygen transport	3.61E-05	0.001	10	8
GO:0005833	hemoglobin complex	3.61E-05	0.001	10	8
GO:0002250	adaptive immune response	3.74E-05	0.001	88	53
GO:0002521	leukocyte differentiation	3.89E-05	0.002	110	60
GO:0009410	response to xenobiotic stimulus	5.10E-05	0.003	10	5
GO:0005885	Arp2/3 protein complex	6.33E-05	0.001	15	15
GO:0030098	lymphocyte differentiation	7.09E-05	0.005	93	35
GO:0007249	I-kappaB kinase/NF-kappaB cascade	8.34E-05	0.003	107	39
GO:0042108	positive regulation of cytokine biosynthesis	9.67E-05	0.003	26	19
GO:0015669	gas transport	0.00011	0.001	11	8
GO:0007596	blood coagulation	0.00014	0.006	65	15
GO:0009620	response to fungus	0.00015	0.001	17	10
GO:0002695	negative regulation of leukocyte activation	0.00019	0.001	19	9

Another group with overlapping genes comprised of six pathways. These were interleukin 8 (IL-8) biosynthesis, positive regulation of IL-8 biosynthesis, IL-8 production, lipopolysaccharide binding, all  $P < 0.001$ , and positive regulation of cytokine biosynthesis  $P < 0.005$ . The overlap between pathways was expected as GOs are built as hierarchical trees where several GOs form a larger ontology. The majority of genes in these pathways consisted of toll-like receptors. The xenobiotic process ( $P < 0.001$ ) and the response to xenobiotic stimulus ( $P < 0.005$ ) as well as I- $\kappa$ B kinase–NF- $\kappa$ B cascade ( $P < 0.005$ ) were also among the significantly enriched. Pro-inflammatory cytokines have been shown to increase after sleep restriction (Irwin et al., 2006, van Leeuwen et al., 2009). These findings complement our results from the GWAS pathways and suggest that inflammatory responses are changed after sleep restriction.

A biologically very different set of GOs was observed among the down-regulated genes (top 30 pathways presented in Table 8). Several pathways participating in lipid transport were enriched: intracellular lipid transport, cholesterol efflux, sequestering of lipids, and sterol transporter activity ( $P < 0.001$ ) being the top ranked. These changes may reflect a compensatory mechanism for short term sleep restriction induced stress as cholesterol levels have been shown to decrease after stress response (Choi et al., 2005). These findings are interesting in the light of previous finding on epidemiological settings where short sleep duration is related to higher circulating blood lipid levels (Kronholm et al., 2011). It is likely that longer sleep deprivation has opposite effects on total cholesterol levels than short term sleep restriction.

In addition, the circadian rhythm pathway was down-regulated ( $P < 0.05$ ). The down-regulation is unlikely to reflect a phase delay effect that was earlier reported to be a modest 16 minutes (van Leeuwen et al., 2010). Rather, it may be caused by dampening of the cortisol and circadian rhythm. Such overall lower amplitude in circadian gene expression has been previously observed with circadian genes (Kavcic et al., 2011). Overall the changes in circadian clock gene expression have been connected to energy, lipid and glucose metabolism at a molecular level (Bass and Takahashi, 2010). Despite the cause, either in amplitude or expression rhythm, this may suggest that the circadian genes are out-of-sync after sleep restriction. It has been proposed that asynchrony in the timing of autonomous internal clocks in the brain and peripheral tissues might contribute to the development of metabolic disease states and experimental studies with shift work and jet lag simulation have supported this hypothesis (Huang et al., 2011).

To summarize, our findings suggest that short sleep duration is related to metabolic and immunological changes in transcriptomic level, which may explain part of the connection with cardiometabolic diseases and sleep. In addition, SNPs in PPAR-signaling pathway and in immunological pathways may predispose to either shorter or longer sleep duration either independently or via comorbid diseases. The

findings with sleep restriction suggest that inflammatory responses may be responsible for some of the changes seen after shorter sleep duration. In addition, metabolic balance may be changed after sleep restriction.

**Table 8. Down-regulated pathways after experimental sleep restriction.** The P-values were calculated as point wise P-values and permuted 1000 times to obtain corrected P-values. The column N genes total shows how many genes are annotated in the pathway. N contributing genes tell how many of the annotated genes are associating in this data set.

ID	Pathway	P	P permuted	N genes total	N contributing genes
GO:0032365	intracellular lipid transport	1.71E-05	0.001	9	4
GO:0015833	peptide transport	0.00015	0.003	20	4
GO:0015918	sterol transport	0.00018	0.001	8	3
GO:0055092	sterol homeostasis	0.00018	0.001	8	3
GO:0030301	cholesterol transport	0.00018	0.001	8	3
GO:0042632	cholesterol homeostasis	0.00018	0.001	8	3
GO:0045736	negative regulation of cyclin-dependent protein kinase activity	0.00029	0.003	9	5
GO:0006869	lipid transport	0.00037	0.006	49	6
GO:0055088	lipid homeostasis	0.00038	0.002	10	3
GO:0005548	phospholipid transporter activity	0.00039	0.006	14	3
GO:0005319	lipid transporter activity	0.00049	0.004	25	4
GO:0015909	long-chain fatty acid transport	0.00058	0.005	9	3
GO:0008299	isoprenoid biosynthetic process	0.00073	0.004	7	7
GO:0032393	MHC class I receptor activity	0.00075	0.005	7	6
GO:0006635	fatty acid beta-oxidation	0.00082	0.01	10	3
GO:0004715	non-membrane spanning protein tyrosine kinase activity	0.00082	0.011	16	11
GO:0050680	negative regulation of epithelial cell proliferation	0.00104	0.012	15	3
GO:0015908	fatty acid transport	0.00111	0.007	11	3
GO:0009062	fatty acid catabolic process	0.00111	0.008	11	3
GO:0004860	protein kinase inhibitor activity	0.00117	0.018	19	5
GO:0016538	cyclin-dependent protein kinase regulator activity	0.00126	0.009	11	5
GO:0048286	alveolus development	0.00128	0.01	7	4
GO:0030509	BMP signaling pathway	0.00129	0.011	13	3
GO:0007623	circadian rhythm	0.00145	0.015	14	12
GO:0046395	carboxylic acid catabolic process	0.00146	0.006	12	3
GO:0016054	organic acid catabolic process	0.00146	0.007	12	3

## 5.2 Genetic Connection between Metabolism and Sleep (II)

Our findings from GWAS pathways and RNA expression in sleep restriction prompted us to look in more detail at the connection between cardiometabolic diseases and sleep at the epidemiological and at the genetic level. Recently it was found that the heritability for BMI and blood lipid composition is higher in individuals having short sleep duration (Watson et al., 2010). This can be explained in two ways. First, short sleep is a risk environment for BMI or lipid genes and second, there may be common genetic variants that predispose both to high BMI and lipid levels as well as to shorter sleep duration.

We characterized the connection between blood lipid levels and sleep duration at the population level in a data set that combined two Finnish population samples, Finrisk07 and Health 2000. We observed a significant association between lipid levels and sleep duration so that both ends of sleep, short and long sleep, had a worse lipid profile. This was tested by comparing the sleep duration groups at the ends (5 and 6 hours) separately to the rest of the sleep duration groups with ANOVA. A similar analysis was also performed separately with the long sleep duration groups (9 and 10 hours) with the rest of the sleep duration groups (Table 9). The association of the high levels of triglycerides (TG), total cholesterol (TC) and low levels of HDL-cholesterol was seen in both short and long sleepers, whereas LDL-cholesterol was lowest in individuals sleeping nine hours or more (Table 9).

**Table 9. Association of lipid levels with sleep duration.** The association between sleep time and lipid variables from Finnish population samples Health 2000 and Finrisk 07. Abbreviations: HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; TC, total cholesterol; TG, triglyceride.

a ANOVA P-value <0.05 indicates significant association with sleep duration when comparing the group to all other sleep duration groups within the lipid variable.

b ANOVA P-value <0.01 indicates significant association with sleep duration when comparing the group to all other sleep duration groups within the lipid variable.

c ANOVA P-value <0.001 indicates significant association with sleep duration when comparing the group to all other sleep duration groups within the lipid variable.

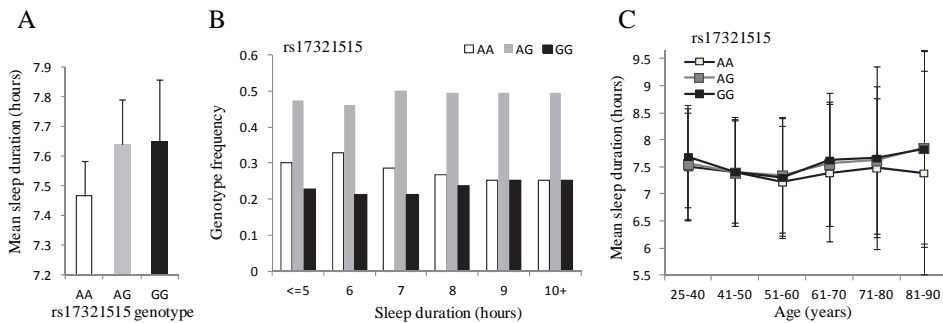
Total sleep time (hours)	N	TG mmol l <sup>-1</sup> ±SD	TC mmol l <sup>-1</sup> ±SD	HDL-C mmol l <sup>-1</sup> ±SD	LDL-C mmol l <sup>-1</sup> ±SD
5	370	1.63±1.04a	5.77±1.08b	1.36±0.41	3.61±1.145a
6	1327	1.57±1.04a	5.65±1.12a	1.38±0.39	3.51±1.11
7	4101	1.48±0.97b	5.61±1.11b	1.39±0.38	3.5±1.08b
8	4472	1.50±0.96	5.57±1.11b	1.39±0.38	3.45±1.09b
9	1208	1.53±1.00	5.48±1.07c	1.39±0.37	3.37±1.01c
10	562	1.79±1.28c	5.57±1.20	1.31±0.39c	3.36±1.17a
Total	12 040	1.52±1.00	5.59±1.11	1.38±0.38	3.47±1.09

We then studied whether there are shared genetic factors that contribute both to the regulation of lipid levels and the regulation of sleep duration. We selected 59 risk variants from GWA studies with blood lipid levels that were published before September 2010 when the selection was made (Teslovich et al., 2010) and performed association analysis with these variants and sleep duration. Two genetic variants associated significantly with sleep duration and the findings sustained correction for multiple testing (rs17321515  $\beta=0.081$ ,  $P=8.92 \times 10^{-5}$ , Bonferroni corrected  $P=0.0053$ ; rs2954029,  $P=0.00025$ , corrected  $\beta=0.076$ ,  $P=0.015$ ). All variants with nominal  $P < 0.05$  are presented in Table 10.

**Table 10.** Association of lipid gene candidate SNPs with sleep duration. *TRIB1* shows significant association with sleep duration (linear model adjusted for age and gender).

CHR	SNP	BP	MAF	BETA	P	GENE
8	rs17321515	126555591	G	0.0815	8.92E-05	<i>TRIB1</i>
8	rs2954029	126560154	T	0.0764	2.46E-04	<i>TRIB1</i>
1	rs4846914	228362314	G	-0.0539	0.0103	<i>GALNT2</i>
7	rs12670798	21573877	C	-0.0582	0.0148	<i>DNAH11</i>
11	rs174570	61353788	T	0.0540	0.0301	<i>FADS3/FADS2</i>
1	rs12740374	109619113	T	-0.0505	0.0480	<i>SORT1</i>

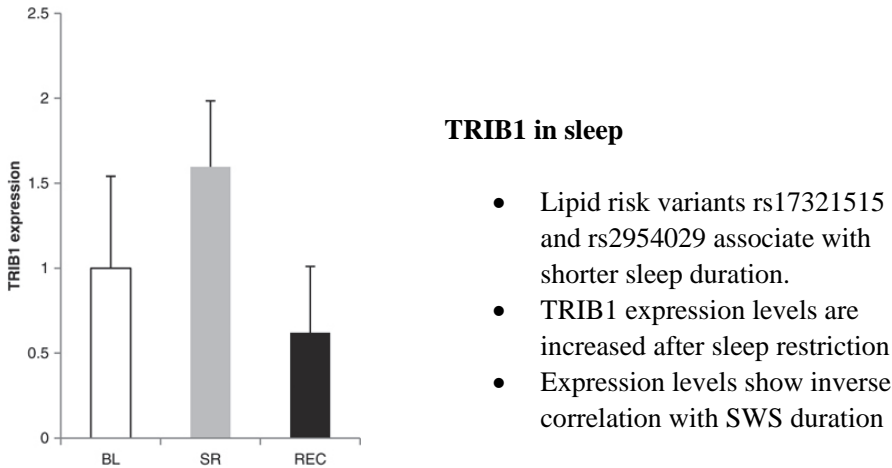
The most significant variant, rs17321515 near *TRIB1* was supported in an independent sample of 2,189 Finnish twins ( $\beta=0.063$ ,  $P=0.022$ ). Meta-analysis of the two Finnish data sets further strengthened the association ( $\beta=0.073$ ,  $P=8.1 \times 10^{-6}$ ), further supporting the role of *TRIB1* in sleep regulation. Analysis of genotype groups revealed that the individuals carrying the protective minor allele for lower blood lipid levels had longer sleep duration (Figure 12A) and the risk variants were more common in shorter sleep duration groups (Figure 12B). The individuals carrying risk genotype AA showed association to the same direction (shorter sleep duration) in all age groups except for those between 41-50 years (i.e. 25-40 years, 51-60 years, 61-70 years, 71-80 years, 81-90 years). Based on OR values it seemed that the *TRIB1* variant would have a larger effect among older individuals. However, excluding individuals that were over 60 years of age did not remove the association even though the significance and the effect size after removing these individuals was lower ( $\beta=0.048$ ,  $P=0.037$ ) (Figure 12 C). As sleep duration and lipid levels are connected in phenotype level it is possible that the effect seen with sleep duration is caused by the association with lipid levels. We thus performed the same association analysis but adjusted the analysis with blood lipid levels. However, adjusting for blood lipid levels did not abolish the association with shorter sleep duration, suggesting that *TRIB1* had an independent role in sleep regulation as well. Similarly, it could be possible that age or comorbidities related to sleep or lipid levels would explain the association. We tested this by excluding individuals with sleep problems, use of hypnotics and use of lipid medication, which did not abolish the association. In contrast, the association was stronger in this group (TG adjusted  $\beta=0.082$ ,  $P=7.82 \times 10^{-5}$ , HDL-C adjusted  $\beta=0.084$ ,  $P=5.77 \times 10^{-5}$ ).



**Figure 12.** Association of *TRIB1* rs17321515 genotypes with sleep duration (A) and genotype frequencies in different sleep duration groups (B). The association was not dependent on age. This is visualized in C where tendency for shorter mean sleep duration was observed in all age groups except for those between 41-50 years for major major (AA) genotype (C). These differences did not reach statistical significance in age group level. It seemed that older individuals would have larger effect. However, excluding individuals that were over 60 years of age did not remove the association.

Finally, we studied the levels of *TRIB1* RNA expression after experimental sleep restriction in a separate study in nine healthy volunteers. Functional evidence of *TRIB1* after sleep restriction showed that the expression of *TRIB1* increased 1.6 fold on average in sleep restriction ( $P=0.006$ , Figure 13). In addition, the baseline normalized RNA expression of *TRIB1* showed inverse correlation with the baseline normalized SWS amount in recovery, suggesting that *TRIB1* may have a role in SWS regulation. Earlier studies on cholesterol levels both in epidemiological and experimental sleep deprivation studies in mice have shown that the transcription of genes regulating cholesterol synthesis and lipid transport increase in sleep (Giebultowicz and Kapahi, 2010). In addition, sleep deprivation studies performed in *Drosophila* have found a relationship between lipid metabolism and tolerance to sleep deprivation, suggesting that changes at the level of enzymes that modulate lipid metabolism enzymes alter the response to sleep deprivation (Thimman et al., 2010). Based on these data we propose that the association between sleep and lipid levels is mediated partially through the same genes such as *TRIB1*.





**Figure 13. TRIB1 levels in sleep restriction.** TRIB1 mRNA levels in sleep restriction. Baseline (BL), sleep restriction (SR) and recovery (REC). BL=1.

TRIB1 is expressed almost in all tissues, with the highest expression being observed in the brain, liver and leukocytes (Hidalgo et al., 2009). Hepatic TRIB1 over expressing mice show lower TG, VLDL, HDL-C, LDL-C levels and APOB secretion. In *Trib1* knockout animals an opposite lipid profile is observed and functional studies suggest the involvement of acetyl-CoA carboxylase 1 (*Acc1*), fatty acid synthase (*Fasn*) and stearoyl-coenzyme A desaturase1 (Hidalgo et al., 2009). No studies of the activity or sleep of these animals have been reported so far. Our findings suggest that the association between sleep duration and *TRIB1* is not directly dependent on the effect of *TRIB1* on lipids.

Another protein in tribbles family, TRIB2 has been related to pathogenesis of narcolepsy and in another autoimmune disease, uveitis (Cvetkovic-Lopes et al., 2010, Zhang et al., 2005). Both TRIB1 and TRIB2 also regulate inflammatory reactions and Akt and MAPK signaling (Hegedus et al., 2007, Kiss-Toth et al., 2004, Kiss-Toth et al., 2006). However, they differ also in regard to their cellular location: TRIB1 has been detected in the nucleus whereas TRIB2 is a cytoplasmic protein. Similarly, in GWA studies *TRIB1* is related to energy metabolism and has been found in lipid trait and coronary heart disease. In contrast, *TRIB2* seems to be related to inflammatory reactions and autoantibody production in narcolepsy and uveitis. Our findings suggest that *TRIB1* variants that associate with both sleep and lipid levels may function in normal sleep regulation. One of the aspects of sleep is to balance changes in energy expenditure or prolonged waking (Benington and Heller, 1995). *TRIB1* could have an effect in these functions as variants of *TRIB1* associate

with both sleep and lipid metabolism as well as correlate with the recovery SWS after sleep restriction.

### 5.3 Genetic Connection of Depression and Genes Related to Adenosine (III)

Sleep and depression are connected at the epidemiological level and poor sleep quality predisposes to depression (Paunio et al., 2009). Individuals suffering from depression have specific sleep problems. Depression, like other neuropsychiatric diseases, is not clearly one disease. Individuals suffering from depression can have a variety of sleep problems like insomnia, or symptoms of insomnia such as early morning awakenings as well as fatigue. It is tempting to consider that the same genetic variants would control sleep and depression. Moreover, the variants affecting depression with early morning awakenings could be distinct from those that affect depression with fatigue. We have previously shown that variants in five distinct neural networks that are related to depression associate with distinct subtypes of depression with either early morning awakenings or fatigue. The associating variants are located in glutamatergic (*GRIA3*, *GAD1*, *P2RX7* and *DAOA*), serotonergic (*SLC6A4* and *TPH2*), neural plasticity (*DISC1*, *CREB1* and *BDNF*) and HPA axis genes (*CRHRI*) (Utge et al., 2011). In addition, we observed that variants in the circadian system, *TIMELESS*, associate with depression and sleep disturbances (Utge et al., 2010). These earlier findings suggest that these variants also regulate aspects of sleep in depression.

Adenosine is one of the best characterized modulators of sleep (Basheer et al., 2004, Porkka-Heiskanen et al., 1997). It modulates sleep intensity via adenosine A1 and A2A receptors (Bjorness et al., 2009, Retey et al., 2005, Urade et al., 2003). Adenosine can also be used to make adenosine triphosphate that is used for energy production (Porkka-Heiskanen et al., 1997). Lack of energy is also a core symptom of depression and it has been suggested that a decreased metabolic rate could explain these symptoms (Tsiouris, 2005). We hypothesized that impaired synthesis or transport of adenosine could be responsible for down-regulation of energy production. Here we wanted to elucidate the role of polymorphisms in genes related to adenosine and study their role in depression.

The genes related to adenosine were studied in the depression sample that had been selected earlier and included all individuals from the Health 2000 project that had a depression diagnosis. The sample comprised of 383 cases and 1,357 controls. It was further divided as depression (N=383, 258 females and 125 males), depression with early morning awakenings (N=109 females, 61 males) and depression with fatigue (N=194 females, 103 males). Altogether 117 SNPs that tagged the coding region and 10kb upstream and 10kb downstream of the coding region in thirteen genes related to adenosine were studied. The genes selected were

adenosine transporters, receptors and metabolic enzymes. Association analysis with depression, symptoms of early morning awakenings and fatigue was performed separately in both genders. Bonferroni correction was used to correct for multiple testing.

The most significant variant that sustained Bonferroni correction was found with depression in females in *ENT3* (*SLC29A3*) with variant rs12256138 (OR=0.68,  $P=0.0004$ ,  $P$  corrected = 0.046). The same variant also associated with early morning awakenings (OR=0.64,  $P=0.0033$ ). Since we already genotyped all individuals that were diagnosed with depression, we studied the association in individuals with early morning awakenings that is a common symptom and risk factor for depression. The same variant was associated in the rest of the females from Health 2000 with early morning awakenings (OR=0.81,  $P=0.043$ , unpublished observations). These data suggest that variation in *ENT3* may have a role in depression and in early morning awakenings, in women.

No variants sustaining Bonferroni correction was found in the analysis of men. Point wise significant findings for both genders were found largely in the *ENT* transporters. However, the only gene that associated in both genders was *ADA* (variant rs6031682 in females and rs452159 for males) that showed point wise association with depression and fatigue. Another *ADA* variant has previously been associated with sleep intensity but it was not genotyped in our sample due to technical problems. It is noteworthy that females consistently show more significant findings in our samples with depression. This may be partially due to larger heritability component of depression in females ( $h^2=0.42$ ) compared to males ( $h^2=0.29$ ) (Kendler et al., 2006). Another contributing factor is most likely the sample size as there were only 109 males diagnosed with depression in the data set compared to 258 females. Overall this study is limited by its small sample size.

Adenosine has previously been related to sleep homeostasis but an increasing amount of literature is emerging of the function of adenosine in depression. In addition to metabolic rate, adenosine is related to neurotransmitter release, of which especially glutamate and dopamine are essential to mood regulation. Depressed individuals show increase in cortical glutamate and adenosine is capable of increasing extracellular levels of glutamate (Boison et al., 2009). Originally we hypothesized that adenosine may be involved in this process via its receptors or via its synthesis. Our current findings suggest however, that variation in the *ENT* transporters that regulate the levels of intra- and extracellular adenosine may play a larger role in the connection between depression and adenosine. However, with the current findings and the small data set, this hypothesis remains speculative. Together these findings suggest that adenosine may be related to depression with sleep disturbances and depression alone. In summary with our previous findings on circadian genes in mood regulation, we can conclude that both circadian and homeostatic factors have an effect on depressive disorder, sleep disturbances and

normal sleep duration. It is also the same variants that mediate all these traits, suggesting that mood and sleep regulation are connected at the genetic level as well.

#### 5.4 Circadian, Seasonal and Cognitive Performance Associates with Bipolar Disorder Risk Variants (IV)

We previously studied the association of 26 SNPs that showed promising association with BD (Baum et al., 2008a) in a Finnish Bipolar family sample of 723 individuals from 180 families (Ollila et al., 2009). The previous association analysis concentrated on BD. The previous study as well as the current study described below, included variants that were not genome-wide significant in the discovery samples. Such suggestive signals were, however, interesting candidate variants and were included in the studies since only one variant for BD with genome-wide significance had been detected at the time when these studies were performed. As also discussed in study I, the variants with suggestive association can be of biological relevance.

In the present study, we aimed to include variants from two additional GWA-studies (Sklar et al., 2008, WTCCC, 2007). In the present study analysis of additional quantitative intermediate phenotypes for bipolar disorder (endophenotypes) that were not included in the previous study, were analyzed in the same Finnish Bipolar family sample. In addition, association analysis with BD was performed for those variants that were not studied in the earlier study in the Finnish sample for BD. Two suggestive associations with BD in the Finnish bipolar sample were observed in the current study (point wise  $P < 0.05$ ). The variants were located at the vicinity of *LPINI* (rs4027132) and *CDH7* SNPs with high LD ( $r^2 > 0.8$  for all variants, from rs2850699, rs2850700, rs2850701, rs2658046, rs976882, rs12970791 and rs1444067). The variants associated in the previous study were at the vicinity of *SORCS2* (rs4411993, rs7683874, rs10937823), *DFNB31* (rs10982256) and *SLC39A3* (rs4806874). The variants in *SORCS2* region were not in LD with each other ( $r^2 < 0.80$ ). The associations from the present study and from the previous study (Ollila et al., 2009) are shown in Table 11.

Previous studies have shown that circadian and seasonal rhythms are often disturbed in BD and changes in these rhythms can induce mania (Jackson et al., 2003). We hypothesized that the same variants that contribute to BD would have an effect on sleep or cognitive endophenotypes for BD. We thus selected the 13 variants that were associated with BD in the Finnish Bipolar family sample (Ollila et al. 2009, study IV) and studied their association with chronotype, seasonality and various domains of cognition. The latter included 22 neuropsychological test variables from the Wechsler Adult Intelligence Scale Revised (WAIS-R), the Wechsler Memory Scale Revised (WMS-R), the California Verbal Learning Test, the Stroop Color and Word Test, and the Controlled Oral Word Association Test.

Analyses with seasonal, circadian and cognitive phenotypes were adjusted for age, gender and BD in order to remove the association signal coming from the disease. The association results for the seasonality traits measured as seasonal changes in sleep duration, social activity, mood, energy level, weight and appetite are presented in Table 12. The associations with the neurocognitive test variables with point wise  $P < 0.05$  are presented in Table 13.

The strongest association signals for the quantitative endophenotypes were obtained with the variant from the previously published replication study in the Finnish bipolar family sample (Ollila et al., 2009), which in the present study associated with seasonal change in sleep duration *DFNB31* (rs10982256, point wise  $P = 0.005$ ). *DFNB31* SNP rs10982256 was also the only SNP that sustained correction for multiple testing in the previously reported replication analysis for BD (Ollila et al., 2009).

Variants in the region of *CDH7* showed association with seasonal and circadian phenotypes as well as with those cognitive tests that were related to visual working memory and visual attention ( $P < 0.01$ ). The most consistent findings were observed with variant rs2850701. Interestingly, the same variants that associated suggestively with increased BD also associated with better performance in visual memory and attention. The function of *CDH7* is related to brain development (Luo et al., 2004) and animal studies have shown that it is expressed in the developing eye and retina (Faulkner-Jones et al., 1999). Our findings suggest that variants in *CDH7* are related to phenotypes that process visual information such as circadian and seasonal changes in light-dark transitions. The study also shows that same variants that predispose to BD may also be beneficial for other traits, such as visual memory or attention. Our findings are consistent with the hypothesis that variants predisposing to psychiatric diseases may have an evolutionary advantage. However, these findings should be interpreted with caution since they were not genome-wide significant in the original discovery samples and were not adjusted for multiple correction in the Finnish data set.

**Table 11. Association with BD and mood disorders.** Association with previously associated variants with BD in *SORCS2*, *DFNB31* and *SLC39A3* by Ollila et al 2009 and associations in the present study *LPIN1* and *CDH7*. BD=BD type I. Mood= any mood disorder. Variants within *CDH7* measure the same signal ( $r^2 > 0.8$  for all variants). Variants within *SORCS2* measure different signals ( $r^2 < 0.80$ ). Positive Z-values indicate association of the minor allele.

Gene	SNP	GWAS	Maj/Min	MAF	Z	P BD	Z	P MOOD
LPIN1	rs4027132 <sup>B</sup>	WTCCC	A/G	0.34	-2.03	0.042	-1.80	0.071
SORCS2	rs4411993 <sup>A</sup>	Baum et al., 2008	C/T	0.19	2.75	0.006	2.46	0.014
SORCS2	rs7683874 <sup>A</sup>	Baum et al., 2008	G/A	0.12	2.49	0.013	2.61	0.009
SORCS2	rs10937823 <sup>A</sup>	Baum et al., 2008	C/T	0.11	2.86	0.004	2.83	0.005
DFNB31	rs10982256 <sup>A</sup>	WTCCC	C/T	0.44	2.58	0.01	3.23	0.001
CDH7	rs2850699 <sup>B</sup>	Sklar et al., 2008	T/G	0.37	-2.34	0.019	-2.20	0.028
CDH7	rs2850700 <sup>B</sup>	Sklar et al., 2008	C/T	0.38	-2.52	0.012	-2.32	0.021
CDH7	rs2850701 <sup>B</sup>	Sklar et al., 2008	T/G	0.38	-2.46	0.014	-2.08	0.038
CDH7	rs2658046 <sup>B</sup>	Sklar et al., 2008	T/C	0.37	-2.31	0.021	-1.76	0.078
CDH7	rs976882 <sup>B</sup>	Sklar et al., 2008	G/A	0.36	-2.24	0.025	-2.06	0.039
CDH7	rs12970791 <sup>B</sup>	Sklar et al., 2008	G/T	0.36	-2.01	0.044	-1.66	0.097
CDH7	rs1444067 <sup>B</sup>	Sklar et al., 2008	C/A	0.38	-2.37	0.018	-2.27	0.023
SLC39A3	rs4806874 <sup>A</sup>	Baum et al., 2008	A/G	0.41	-2.12	0.034	-1.13	0.259

<sup>A</sup> Association with BD reported in Ollila et al 2009

<sup>B</sup> Selected for the current study IV

**Table 12. Association with seasonality phenotypes: change in sleep duration, social activity, mood, weight and appetite between seasons.** The associations with traits showing point wise association of P<0.05 are shown. Association with chronotype (morningness eveningness), seasonal changes in social activity, mood, weight, appetite and energy levels. Positive Z values indicate association of minor allele and negative values indicate association of major allele.

Gene	Seasonality											
	Sleep duration						Social activity			Mood		
	SNP	Z	P	Z	P		Z	P		Z	P	
LPIN1	rs4027132	-1.025	0.305	-0.867	0.386		-0.735	0.462		-1.939	0.053	
SORCS2	rs4411993	1.263	0.207	-0.168	0.866		0.664	0.507		-0.775	0.438	
SORCS2	rs7683874	-1.996	0.046	—	—		—	—		—	—	
SORCS2	rs10937823	1.996	0.046	—	—		—	—		—	—	
DFNB31	rs10982256	2.784	0.005	1.615	0.106		2.588	0.010		2.674	0.008	
CDH7	rs2850699	-1.743	0.081	-2.099	0.036		-1.863	0.062		-1.740	0.082	
CDH7	rs2850700	-2.011	0.044	-2.244	0.025		-2.095	0.036		-1.694	0.090	
CDH7	rs2850701	-2.026	0.043	-2.384	0.017		-2.200	0.028		-1.844	0.065	
CDH7	rs2658046	-1.689	0.091	-2.108	0.035		-1.761	0.078		-1.664	0.096	
CDH7	rs976882	-1.644	0.100	-2.017	0.044		-2.148	0.032		-1.657	0.098	
CDH7	rs12970791	-1.521	0.128	-1.897	0.058		-2.102	0.036		-1.397	0.162	
CDH7	rs1444067	-1.793	0.073	-2.017	0.044		-2.148	0.032		-1.833	0.067	
SLC39A3	rs4806874	0.549	0.583	0.351	0.726		1.335	0.182		0.703	0.482	

**Table 13.** Association of bipolar candidate SNPs with intermediate phenotypes verbal working memory, visual attention and visual working memory. Positive  $\beta$  values indicate association of minor allele. Associations with with point wise  $P<0.05$  are shown.

Gene	SNP	Circadian clockwork			Visual attention			Visual working memory			Verbal working memory							
		β	P	Plink	P	QTD	P	β	P	Plink	P	QTD	P	β	P	Plink	P	QTD
LPIN1	rs4027132	1.246	<b>0.037</b>	0.061	-0.313	0.214	<b>0.045</b>	-0.287	0.352	0.140	-0.419	0.140	<b>0.015</b>					
SORCS2	rs4411993	0.865	0.250	0.287	0.177	0.523	0.741	0.094	0.766	0.945	0.158	0.607	0.592					
SORCS2	rs7683874	-0.359	0.698	0.649	-0.347	0.309	0.179	-0.048	0.901	0.717	-0.276	0.467	0.717					
SORCS2	rs10937823	-0.359	0.698	0.652	-0.404	0.241	0.168	-0.126	0.748	0.668	-0.295	0.443	0.731					
DFNB31	rs10982256	-1.094	0.062	0.091	-0.164	0.405	0.753	0.044	0.844	0.634	-0.173	0.429	0.491					
CDH7	rs2850699	-1.144	0.053	<b>0.035</b>	-0.648	<b>0.013</b>	<b>0.008</b>	-0.627	0.050	<b>0.020</b>	0.050	0.866	0.356					
CDH7	rs2850700	-1.170	0.057	<b>0.040</b>	-0.667	<b>0.011</b>	<b>0.010</b>	-0.657	<b>0.043</b>	<b>0.019</b>	0.038	0.900	0.360					
CDH7	rs2850701	-1.172	0.053	<b>0.039</b>	-0.687	<b>0.010</b>	<b>0.006</b>	-0.673	<b>0.040</b>	<b>0.017</b>	0.021	0.945	0.314					
CDH7	rs2658046	-1.146	0.058	0.053	-0.663	<b>0.013</b>	<b>0.009</b>	-0.636	0.052	<b>0.019</b>	0.040	0.896	0.335					
CDH7	rs976882	-1.137	0.059	<b>0.047</b>	-0.683	<b>0.008</b>	<b>0.006</b>	-0.656	<b>0.042</b>	<b>0.019</b>	0.030	0.920	0.334					
CDH7	rs12970791	-1.328	<b>0.022</b>	<b>0.010</b>	-0.547	<b>0.034</b>	<b>0.016</b>	-0.577	0.069	<b>0.022</b>	0.161	0.585	0.512					
CDH7	rs1444067	-1.147	0.055	<b>0.042</b>	-0.684	<b>0.010</b>	<b>0.006</b>	-0.667	<b>0.041</b>	<b>0.016</b>	0.013	0.965	0.309					
SLC39A3	rs4806874	0.219	0.683	0.665	-0.025	0.901	0.620	0.415	0.068	0.261	-0.059	0.792	0.987					



## 6 Concluding Remarks and Future Prospects

This thesis aimed to characterize genetic variants that on the first hand contribute to normal sleep duration and on the other hand relate sleep duration and sleep disturbances with co-morbidities. In addition to a traditional GWA study a functional approach was used that combined RNA expression in population level and from experimental sleep restriction together with the original GWA study. We found suggestive association with a variant rs2031573 near *KLF6* transcription factor, which associated with shorter sleep duration in the discovery sample and showed suggestive point wise association in the follow-up sample. While the association of this variant did not reach genome-wide significance, its relation to sleep was supported by functional evidence. Increased *KLF6* expression associated with shorter sleep duration in a population-based sample. In addition, increased gene expression was also observed in an independent sample where sleep duration was experimentally restricted. Similarly, the expression levels of *KLF6* associated with increased SWS duration. This association may be mediated through iNOS signalling since iNOS has a *KLF6* binding site in its promoter region. These findings are consistent with that variants near *KLF6* may contribute to sleep duration via iNOS mediated signaling. Since only two genome-wide association studies of sleep have been carried out so far and only one variant with genome-wide significance has been detected, more studies with larger sample size are needed in order to dissect the genetic factors in sleep regulation. Methodologically, our approach suggests that combining functional analysis with GWA studies, even with a relatively small sample size may be informative especially in the field of sleep research where precise phenotypes in population level can be hard to measure.

This thesis evaluated the mechanism that connects short sleep and insufficient sleep amounts with cardiometabolic diseases. Short sleep duration is a risk factor for developing T2DM and obesity. We observed activation of immune reaction, down regulation of lipid transport and synthesis, and down-regulation of circadian pacemaker genes after experimentally induced sleep restriction. The data suggest that the activation of low-grade inflammation together with metabolic changes in cholesterol and lipid synthesis take place in sleep restriction. These changes may help to cope with short-term sleep debt but are likely to induce pathological changes in the long term. In contrast, we found that in population sample short sleep duration was related to increased blood cholesterol levels. It is likely that longer sleep deprivation has opposite effects on total cholesterol levels than short term sleep restriction. These findings may explain the well-established connection between

sleep and cardiometabolic diseases but the mechanisms controlling these changes remain still largely unexplored and need to be studied further.

At the genetic level we studied the role of common genetic variation of sleep in somatic and psychiatric diseases. The variants in *TRIB1* were associated with sleep and lipid traits. The association with sleep duration was independent from lipid levels, suggesting that *TRIB1* polymorphisms may have an independent role in sleep regulation. These findings were supported by two observations: 1) *TRIB1* RNA levels were increased after experimental sleep restriction and 2) the expression change in *TRIB1* RNA expression levels associated with the changes in SWS duration.

In the depression study we found that variants in adenosine metabolism associate with depression. The strongest finding was observed with *ENT3* in females and it sustained multiple testing. The other most significant findings came with the adenosine transporters as well, rather than with the enzymes that catabolise adenosine. These data suggest that the adenosine transporters may have a larger role in the aetiology of depression than what has been thought before.

In the association study with BD, we observed a genetic connection with chronotype and visual memory with *CDH7* genotypes. Individuals with BD have a more variable circadian rhythm (Jones et al., 2005). Previously, variants in the circadian pacemaker genes have also been associated with BD (Benedetti et al., 2007, Nievergelt et al., 2006, Soria et al., 2010). The phenotypes that were related to chronotype and seasonality were also associated with *CDH7*. In addition, the *CDH7* variants that associated with cognitive tests were associated also with visual attention and visual memory, traits that all are related to visual processing of light. Previous studies of *CDH7* have shown that it regulates the development of eye and retina (Faulkner-Jones et al., 1999, Luo et al., 2004). Together with previous findings our data suggest that the variants in *CDH7* may be connected with BD through their role in light-dark transitions. These findings suggest that sleep and psychiatric diseases are closely connected also on the genetic level.

Interestingly, we were able to show that those individuals who carried the risk genotype of *CDH7* for BD performed better in cognitive testing. These findings support the hypothesis that not all risk variants alone are malignant but require possibly other risk variants or specific environmental exposure. As noted earlier, risk factors for one trait can be beneficial for another trait. These themes should be considered more in detail in the field of psychiatric genetics.

In these studies we used related phenotypes and endophenotypes to divide the study subjects. This was done in order to obtain phenotypically, and potentially also genetically more homogenous population. The data suggest that genetic variants in both psychiatric and somatic traits associate both with sleep traits and the disease phenotypes. The traits may either be comorbid or one may predispose for the other such as short sleep to metabolic diseases also on the genetic level or dyssynchrony

of circadian rhythm for BD. However, longitudinal studies as well as interventions will be important in revealing the causality of these associations. Our findings support using endophenotypes together with the disease phenotype in order to clean the phenotype. This approach will be useful especially with psychiatric diseases that contain a myriad of phenotypic interactions and present a mix of different phenotypic combinations. In addition, healthy individuals can be used to dissect normal variation with the phenotype. The findings presented in this thesis should, however, be interpreted in the light of following limitations. The discovery samples in the study were relatively small and had limited power to detect variants with genome-wide significance. Larger studies are thus needed in order to detect such variants. The phenotypes in the population cohorts were measured with questionnaires. In the case of sleep duration it is thus hard to distinguish between individuals who are natural short sleepers and who are sleep deprived. These limitations were partially overcome by combining the analyses with functional evidence and experimental sleep restriction. This study would have benefited from functional knock out animal models that could have been used to study the effects of the associating genes with sleep recordings.

The relatively recent advantages in the development of technology in genetics have provided us with a growing number of tools to study complex genetic traits as well. This thesis has aimed to also use unconventional methods for integrating all possible material available. Surely also future advantages in exome sequencing studies will provide us with an even more thorough understanding of the enigmatic traits.

# 7 Acknowledgements

This work was carried out at the Public Health Genomics Unit, National Institute for Health and Welfare (THL) and at the Department of Biomedicine and Physiology at the Faculty of Medicine, University of Helsinki, during the years 2008-2012. I wish to express my deepest gratitude to the heads of the departments Dr. Pekka Puska, Dr. Anu Jalanko, Dr. Esa Korpi and Dr. Antti Pertovaara for providing excellent research facilities and an encouraging scientific research environment.

I want to sincerely acknowledge all the funding sources for making this work possible. I have obtained financial support from the Instrumentarium Science Foundation, Jalmari and Rauha Ahokas Foundation, the Finnish Brain Foundation, Biomedicum Helsinki Foundation, Emil Aaltonen Foundation, the Finnish Foundation of Cardiovascular Research, Psychiatry Graduate School, Paavo Nurmi Foundation, University of Helsinki Funds and the National Institute for Health and Welfare.

In addition, I wish to express my warmest thanks to the Helsinki Graduate Program in Biotechnology and Molecular Biology. I am deeply grateful to Professor Pekka Lappalainen, Dr. Erkki Raulo and Anita Tienhaara at the graduate school for their valuable support and education during the thesis project.

I am deeply grateful and honoured that Professor Debra Skene accepted the role of the Opponent. Also, I am forever grateful to docents Elisabeth Widén and Tarja Saaresranta for the thorough and careful revision of my thesis, and for their constructive suggestions to improve the manuscript. I also want to thank Elisabeth Widén as well as Carina Holmberg-Still for their role in the thesis committee.

I am deeply grateful to my supervisors Dr. Tiina Paunio and Dr. Tarja Porkka-Heiskanen. I was honoured to work with you. Tiina, I value your energy, expertise from clinical work and skills in the field of psychiatric genetics. Tarja, I admire your knowledge of sleep physiology, numerous international connections and aim for perfection in science.

This work would not have been possible without a number of collaborators to whom I am grateful and indebted. Professor Jaakko Kaprio, professor Veikko Salomaa, professor Terho Lehtimäki, professor Olli Raitakari, professor Markus Perola and Matti Jauhiainen are thanked for their support and expertise in genetics. Especially, I wish to thank professor Samuli Ripatti as well as Johannes Kettunen and Ida Surakka in his research group for invaluable contribution and for always finding time to explain details in statistics to me. In addition, Dr. Timo Partonen and Dr. Erkki Kronholm are thanked for their valuable comments, thorough discussions during the thesis project and their expertise in sleep and psychiatry. I also wish to thank all members of the bipolar research team.

My warmest thanks go to the Sleep Team Helsinki and Sleep Mood group members: Vilma Aho, Jukka Alasaari, Natalia Gass, Jenni Kauppinen, Sari Laakkonen, Markus Lagus, Leena Laine, Mackenzie Lind, Johanna Liuhanen, Ernst Mecke, Eeva Palomäki, Marja-Riitta Rautiainen, Emilie Rydgren, Kirsi-Marja Rytönen, Pirjo Saarelainen, Amy Sanders, Sergey Saveljev, Pia Soronen, Johanna Suhonen, Sonja Sulkava, Auli Toivola, Anna Sofia Urrila, Siddheshwar Utge, Maria Volodina, Henna-Kaisa Wigren and Antti-Jussi Ämmälä. Thank you all so much!

I want to thank all the people from psyko, lipid and saattohoito rooms that introduced me into the lab and worked with me during the first years: Olli, Annu, Marika, Pia, Helena, Tea, Ansku, Jarkko and PP. I have had such surreal and fun years in the lab!

I have been exceptionally lucky to work with a number of talented people. During the thesis project many have become my friends. I wish to thank Diana, Niina, Emilia, Johannes, Kati, Marine, Virpi, Tiia, Jussi, Antti-Pekka, Minttu, Karola, Ida, Emmi, Outi, Tero, Pekka, Teppo, Himanshu, Will, Sampo, Kaisa, Mari K and Mari R, Anu, Matti, Aija, Pietari, Kristiina, Katja, Nora, Taru, Joni, Heidi, Iris, Jesper, Kaisu, Juho, Mervi, Päivi, Saana, Suvi, Perttu, Marjis, Krista, Outi, Kaisu, Mervi, Anni, Kirsi, Essi, Peter W and Peter W, Luca, Mikko, Jaakko, Emma, Alfredo, Annina, Minna, Liisa, Tintti and Verner. It was great to escape to BM2 for some coffee and high quality discussions. Sometimes it has taken more than just one song of Tiktak for the science to sink in. Thank you. I also thank all the great people I met during the graduate school years. Special thanks goes also to the secretaries Anne, Irina, Leena, Sari, Liisa and Tuija for making the bureaucratic part of the work easy.

I sincerely thank all the past and present brothers in arms from the fifth floor. My deepest gratitude goes to Terhi Kärpänen. I learned a lot from you. I also wish to thank Michael, Caroline, Antti, Juulia, Marika, Pia-Riitta, Sanna and Heikki, Hanna, Jarkko, Camilla, Marja, Marko, Kaisa, Kukka, Samu, Paula, Maria, Miia, Juho and Wolf. Your friendship and support has made all the difference.

I want to thank all my friends. I thank Annariikka and Ville for the scientific and non-scientific adventures, and for sharing a few fenix drinks during the thesis process. Ilona, Heini, Pauliina and Matti are thanked for dragging me out of the lab once in a while. I also thank all the people from Albertinkatu: Anni, Mikko, Kaisa, Venla and Iina. I had great times with you! My geek and climbing friends from #kaytanhousoja and #climbing are thanked for breaking my fingers either by climbing or typing.

I want to thank my grandparents Antti, Jorma and Pirkko as well as my parents Kaarina and Jukka Ollila. Thank you for teaching me to trust my intuition, for encouraging me to work on a field I found interesting and for creating the soundtrack for my life.

Finally, I want to thank Otto. Thank you for loving me.

Helsinki December 12<sup>th</sup>, 2012

Hanna M. Ollila

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